ISOLATION OF MYCOBACTERIUM TUBERCULOSIS IN BODY FLUIDS AND COMPARISON OF CONVENTIONAL METHODS WITH POLYMERASE CHAIN REACTION IN A TERTIARY CARE HOSPITAL, CHENNAI

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. (MICROBIOLOGY)

BRANCH – IV

GOVT.STANLEY MEDICAL COLLEGE & HOSPITAL

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY CHENNAI, INDIA.

APRIL 2011

DECLARATION

I solemnly declare that this dissertation **"ISOLATION OF MYCOBACTERIUM TUBERCULOSIS IN BODY FLUIDS AND COMPARISON OF CONVENTIONAL METHODS WITH POLYMERASE CHAIN REACTION IN A TERTIARY CARE HOSPITAL-CHENNAI"** is the bonafide work done by me at the Department of Microbiology, Govt. Stanley Medical College and Hospital, Chennai, under the guidance and supervision of **Prof. Dr.R.SELVI, M.D.,** Professor and Head, Department of Microbiology, Govt. Stanley Medical College, Chennai.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in April 2011.

Place : Chennai.
Date :

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CERTIFICATE

This is to certify that this dissertation entitled **"ISOLATION OF MYCOBACTERIUM TUBERCULOSIS IN BODY FLUIDS AND COMPARISON OF CONVENTIONAL METHODS WITH POLYMERASE CHAIN REACTION IN A TERTIARY CARE HOSPITAL-CHENNAI"** is the bonafide original work done by **Dr.N.JAYAMANI,** Post graduate in Microbiology, under my overall supervision and guidance in the Department of Microbiology, Government Stanley Medical College & Hospital, Chennai-600 001, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

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ACKNOWLEDGEMENT

I am extremely grateful to Honorable Dean **Prof. DR.C.VAMSADHARA., M.D., Ph.D.,** Government Stanley Medical College, Chennai for permitting me to carry out this study.

My sincere thanks and appreciation are due to my guide **Prof. Dr. R.SELVI, M.D.,** Department of Microbiology, Stanley Medical College for her guidance and vital support to carry out this study in Department of Microbiology, Stanley Medical College, Chennai.

I express my sincere thanks to former HOD **Prof.Dr.P.R.THENMOZHIVALLI M.D.,** for the valuable guidance.

I owe my debt to **Dr. N.SELVA KUMAR,** Deputy Director (Senior Grade), Head of the Department Tuberculosis Research Centre (ICMR) Chennai, for providing me the opportunity to carry out my dissertation work at TRC and also for his interest and encouragement throughout the period of bacteriological work at TRC. I express my respects and thanks to him.

I express my sincere thanks to **Dr. B. SEKAR, M.D.,** Deputy Director, Centre for Leprosy for teaching and Research Institute for his guidance.

I would like to express my sincere gratitude and heartful thanks to **Dr.VASUMATHI, Dr.DILLI RANI, Dr.USHA KRISHNAN,**

Dr. AGATHA, Dr. SWARNA Assistant Professors of Microbiology for their help and valuable suggestions throughout this study.

My sincere thanks to **Dr.PARAMESWARI M.Sc(Stat).,Ph.D.,** of Balaji Medical College, Chennai for the Statistical guidance for the Study.

My sincere thanks to **T. NAGARAJ,** Senior Lab Technician Officer and Other Technical Staff, TRC, Chennai. My Sincere thanks to **Mr. ARUNAGIRI,** Technical Assitant, Centre for Leprosy for teaching and Research Institute. My heartful thanks to Lab Technicians Stanley Medical College, Department of Microbiology, Chennai.

I express my thanks to all my Post Graduate colleagues.

I would like to thank the institutional ethical committee for approving my study. I also extend my thanks to all the patients who participated in my study.

I am extremely thankful to my husband and children who nurtured and faithfully supported me throughout this study. I dedicate this book to my family.

And all above I thank God who has been with me at all times.

CONTENTS

INTRODUCTION

Tuberculosis has co-existed with humanity since the days before recorded history and evidence of tuberculosis has been found in the skeletal remains of mummies [1]. Hippocrates not only described the disease but also named it 'phthisis' which means to mart or waste away.

Tuberculosis remains a major global public health problem. Roughly a third of the world's population has been infected with *M. tuberculosis*, and new infections occur at a rate of one per second. However, not all infections with *M. tuberculosis* cause TB disease and many infections are asymptomatic. As per WHO 2009 report, in 2008, there were an estimated 8.9-9.9 million incident cases of Tuberculosis [64], 9.6-13.3 million prevalent cases of Tuberculosis 1.1-1.7 million deaths from Tuberculosis.

India had the largest total incidence, with an estimated 2.0 million new cases. India has the most number of cases of tuberculosis and accounts for about one-fifth of the global TB burden and 2/3th of the South –East Asian countries burden. Nearly 40% of the Indian population is affected with tuberculosis bacillus. Each year, 1.9 million cases of TB are detected, amongst which 0.8 million are smear positive new cases (infectious cases) [62]. Daily, nearly 1000 people die due to TB (2 deaths/ 3 minutes). As per WHO estimate, there were nearly 322,000 TB deaths in 2006 in India which accounted for 26% of all the preventable adult deaths.

Socio –economic burden in India – Apart from the considerable morbidity and mortality it causes, TB imparts a great socio – economic burden on India. TB affects people in the age group which is most productive in the population (15-54 years). This disease is even more common among the poorer section of the community and those with a lower socio-economic status. Two-third of the affected population is males, and more than 50% of affected females are below the age of 34.

Tuberculosis, a reemerging killer, is threatening to assume serious proportion all over the world, particularly in view of the AIDS pandemic. Extra -pulmonary tuberculosis is on the increase world over. In countries with good surveillance data like the USA, where the rate of pulmonary tuberculosis has declined to its lowest levels ever in 2001 statistics indicate a relative increase of extra-pulmonary cases from 16% in 1992 to 20% in 2000[9], while its prevalence is 15-30% in immuno-competent persons.

In India, EPTB comprises 20% of all TB cases. Its prevalence in the country varies between 8.3-13.1% in different districts according to cohort analysis by Central TB Division, Ministry of Health & Family Welfare in 2002[13]. In the year 2006, 1,83,180 EPTB cases were registered in comparison to 5,55,660 smear positive pulmonary TB cases giving a ratio of 1:0.24 to 1:0.06. Cure of infectious cases is likely to have resulted in a relative rise of annual EPTB case detection[13]. Prevalence of EPTB has also been found to be higher in pediatric cases[7].

Extra pulmonary tuberculosis can occur alone or in combination with the pulmonary Tuberculosis. It is usually confined to a single site but disseminated form may also occur. Tuberculosis of meninges, spine, nervous system, abdomen, pleura, pericardium, bones and joints are considered severe forms compared to other sites.

Diagnosis of extra pulmonary tuberculosis is difficult. The diagnosis is confirmed by demonstrating AFB using conventional microscopy which is specific and rapid but its sensitivity is less. The microscopy detects positive smear if at least 10000 bacilli be present per ml of sample and culture isolation is possible only if 10-100 bacilli is present.

While culture is more sensitive and specific it takes 6 weeks to get the results. The specimen being a paucibacillary condition, the yield of positive culture has not been high. However using appropriate concentration techniques, multiple medias, and subculture it is made possible to increase the isolation rate in culture.

However, the rapid methods like, radiometric growth detection system in liquid medium and recent molecular techniques such as polymerase chain reaction and restricted fragment length polymorphism (RFLP) have high sensitivity and play a major role in diagnosis of extra pulmonary tuberculosis. PCR techniques involve a number of different targets including IS6110 insertion sequences. The Insertion sequence of IS6110 is a mobile genetic

element and has universal acceptance, since it is found only in mycobacterium tuberculosis complex, M.tuberculosis strain carries multiple copies of the element. Hence this study compares conventional methods with rapid molecular detection of mycobacterium tuberculosis in extra pulmonary specimens.

The World Health Organisation defines Multi-Drug Resistant TB (MDR-TB) as resistance to at least two of the first line drugs used: rifampicin and ionized. It is, by and large, caused when patients default on treatment, though it is also known to be caused by spontaneous mutation of the bacteria as well. The world is also concerned now about XDR-TB or extensively Drug Resistant TB, a subset of MDR-TB also resistant to fluoroquinolones and one of the three injectables, Kanamycin, Capreomycin and Amikacin. XDR-TB has been noted as an emerging health threat, especially in countries like India, with a high prevalence of HIV. Tuberculosis Control India, the wing of the Union Ministry of Health that implements the Revised National Tuberculosis Control Programme (RNTCP), indicates that MDR-TB levels in the country are about three per cent in new cases and 12-17 per cent in re-treatment cases.

WHO said drug-resistant TB spreading faster than ever. Globally there are 500,000 new cases of drug resistance TB every year, about five per cent of the nine million new TB cases.[62]

Data on drug sensitivity pattern would need to be generated to facilitate formulation of proper treatment polices which I have included in my study. Prompt and accurate diagnosis and effective treatment are essential for good patient care and also help in effective treatment of tuberculosis control[36.]

AIMS AND OBJECTIVE

- Study of prevalence of extra pulmonary tuberculosis in a Tertiary Care Hospital, Chennai

- Comparison of smear by Ziehl-Neelsen and Auramine Phenol Fluorescence Staining

- Isolation of Mycobacterium in Extra Pulmonary specimens by employing conventional bacteriological methods.

- Comparison of culture by Kirchner's and Lowenstein-Jensen Medium

- Identification of Mycobacteria by employing standard phenotypic methods.

- Performing drug susceptibility on the isolates identified as Mycobacterium tuberculosis.

- Comparison of conventional methods with molecular method IS 6110 based Polymerase Chain Reaction.

REVIEW OF LITERATURE

TUBERCULOSIS

Tuberculosis, one of the oldest diseases known to affect humans is caused by the bacteria, Mycobacterium tuberculosis complex. The disease usually affects the lungs, although in up to one third of the cases other organs are also involved. If properly treated, virtually all cases of tuberculosis are curable. If untreated, the disease may be fatal within five years in more than half of the cases. Transmission usually takes place through the air borne spread of droplet nuclei produced by patients with pulmonary tuberculosis.

HISTORY

Robert Koch first identified the tubercle bacilli in 1882[53]. In his classic reports he defined the staining procedures for the direct observation of bacilli in clinical specimen. Culture techniques on solid medium for the in-vitro passage of bacilli isolated from clinical or experimental lesions and subsequent inoculation of guinea pigs with cultural materials to confirm its etiologic role in tuberculosis. This became the basis of 'Koch's postulates' the standard criteria for etiologic research in infectious diseases.

Tuberculosis lymphadenitis is the commonest type of TB described in Hippocrates writing dating back to 460-377 BC. It was initially thought to be cured by the touch of the king (King's evil) in Europe.

ETIOLOGIC AGENT

Mycobacteria belong to the Family: 'Mycobacteriaceae' and the order 'Actinomyectales' The name 'Mycobacterium' was given to this genus by Lehmann and Neumann in 1896 on account of the mould like pellicles produced by these bacteria when grown in liquid media.

There are about 100 mycobacterial species. The term tubercle bacilli broadly include the species of mycobacteria which gives rise to tuberculosis in man. These are M. Bovis, M. africanum , M. tuberculosis, M. microti and M .carnetti[17].

The most frequent and important agent of human disease is mycobacterium tuberculosis. It is a thin rod shaped, non-capsulated non-sporing aerobic, bacteria measuring about 0.5μm/3μm in size acid fast and non-motile.

An important character of mycobacteria is their ability to resist decolorization by a weak mineral acid such as 20% sulphuric acid or 3% hydrochloric acid after being stained by an arylmethane dye such as basic fuschin with which it form stable complexes. This is acid fastness and it is due to the presence of mycolic acid in their cell wall.

Upon culture, on Lowenstein Jensen media Mycobacterium tuberculosis form the rough, tough and buff colored colonies after an average incubation period of 2-4 weeks. On liquid kirchner s media which is

incubated up to a maximum period of six weeks, mycobacteria form fine granular growth.

CELL WALL STRUCTURE

The cell wall structure of *Mycobacterium tuberculosis* deserves special attention because it is unique among prokaryotes, and it is a major determinant of virulence for the bacterium. The cell wall complex contains **peptidoglycan**, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of MTB's cell wall consists of three major components, mycolic acids, cord factor and wax-D.

Mycolic acids are unique alpha-branched lipids found in cell walls of *Mycobacterium* and *Corynebacterium.* They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic Acids are thought to be a significant determinant of virulence in MTB. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum.

Cord Factor [63] is responsible for the serpentine cording mentioned above. Cord factor is toxic to mammalian cells and is also an inhibitor of

PMN migration. Cord factor is most abundantly produced in virulent strains of MTB. **Wax-D** in the cell envelope is the major component of **Freund's complete adjuvant** (CFA).

EPIDEMIOLOGY

Epidemiological studies on tuberculosis are concerned with the transmission of the disease in the community and the impact of the control measures. It is estimated that there were 8.8 million new cases of tuberculosis in 2002 of which 3.9 million were smear positive. The global incidence rate of tuberculosis is growing at approximately 1.1% per year and the number of cases at 2.4% per year.

According to WHO, estimates reach 16-20 million cases of tuberculosis worldwide in 2001.It is estimated that about one third of the current global population is infected asymptomatically with tuberculosis of whom 5-10 percent t will develop clinical disease during their lifetime. Most new cases and deaths occur and developing countries where infection in often acquired in childhood.

India accounts for nearly one – third of global burden of tuberculosis[57]. Every year, approximately 1.8 million persons developing tuberculosis of which about 0.8 million are new smear positive highly infection cases and about 4.1 lakh people die of TB every year. One person dies every minute.

The incidence of TB in HIV infected persons is more than 100 times that of the general population. One of the most threatening features of TB in HIV infected patients has been the spread of multidrug - resistant (MDR) organisms. The national AIDS policy documents from Govt. of India show that > 60% of AIDS patients suffer from TB as an opportunistic infection.

SOURCE OF INFECTION:

It is well established that, sputum positive patients i.e. positive on direct microbiological examination and thus contains at least 10000 bacilli in 1 ml and who has either received no treatment or not being treated fully, can discharge the bacilli in their sputum for years. They become the potential sources of infection.

TRANSMISSION AND DEVELOPMENT OF DISEASE

Almost all M. tuberculosis infection is acquired by the inhalation of aerosolized droplet nuclei (1-5μm), which reach the pulmonary alveoli. The probability that a person will become infected depends upon the duration of exposure to the source, the size of the bacillary inoculums inhaled, and the infectivity of the Mycobacterial strain. The probability of an immuno-competent host developing active TB after M. tuberculosis infection is 5-10% over the person's lifetime. Patients with extra pulmonary tuberculosis or

smear negative tuberculosis constitute a minimal hazard for transmissions of infections.

The development of disease depends upon the closeness of the contact, extent of disease, sputum positively of the source case and host parasite relationship. Thus the incubation period may be weeks, months or year.

MYCOBACTERIUM TUBERCULOSIS COMPLEX

The term M. tuberculosis complex refers to a group of very closely related species. All of them cause tuberculosis, a chronic granulomatous disease affecting man and many other mammals.

M. tuberculosis - causes human infection

M. bovis - affects cattle and other mammals

M. microti - pathogen of voles and other smaller mammals

M. africanum - intermediate between human & bovine types.

NON- TUBERCULOUS MYCOBACTERIA

During the early 1950s, after it had become routine practice to culture clinical specimen for M. tuberculosis, it was realized that other mycobacteria can also cause disease in human. These organisms became known collectively as non-tuberculous mycobacteria. The non-tuberculous

mycobacteria, especially the Mycobacterium avium complex and M. scrofulaceum should be kept in mind in the HIV millennium.

EXTRAPULMONARY TUBERCULOSIS

Extra-pulmonary tuberculosis refers to disease outside the lungs, in developed countries, 10-15% of TB cases have extra-pulmonary involvement, but in patients from high-incidence countries the rate is much higher. People who are HIV positive and infected with TB develop extra-pulmonary disease much more frequently, up to 50% of cases.

TUBERCULOUS PLEURAL EFFUSION

The current hypothesis for the pathogenesis of primary tuberculous pleural effusion is that a subpleural caseous focus in the lung ruptures into the pleural space6–12 weeks after a primary infection[49]. Mycobacterial antigens enter the pleural space and interact with T-cells previously sensitized to mycobacteria, resulting in a delayed hypersensitivity reaction and the accumulation of fluid. It seems that this reaction of the pleura augments the entry of fluid into the pleural space by increasing the permeability of pleural capillaries to serum proteins [28], and thereby increasing the oncotic pressure in the pleural fluid. Involvement of the lymphatic system probably also contributes to the accumulation of pleural fluid. An impaired clearance of

proteins from the pleural space has been reported in human tuberculous effusions [26]. It is known that the clearance of proteins and fluid from the pleural space is carried out by lymphatics in the parietal pleura. Fluid gains access to the lymphatics through openings in the parietal pleura called stomata[60]. Since the parietal pleural is diffusely affected with pleural tuberculosis, damage to or obstruction of the stomata could be an important mechanism leading to accumulation of pleural fluid.

TUBERCULOSIS OF ABDOMEN

The postulated mechanisms by which the tubercule bacilli reach the gastrointestinal tract are: (*i*) hematogenous spread from the primary lung focus in childhood, with later reactivation; (*ii*) ingestion of bacilli in sputum from active pulmonary focus; (*iii*) direct spread from adjacent organs; and (*iv*) through lymph channels from infected nodes. The earlier belief that most cases are due to reactivation of quiescent foci is being challenged with a recent study using DNA fingerprinting showing that 40 per cent cases are due to reinfection. In India, the organism isolated from all intestinal lesions has been *Mycobacterium tuberculosis* and not *M.bovis*. The most common site of involvement is the ileocaecal region, possibly because of the increased physiological stasis, increased rate of fluid and electrolyte absorption, minimal digestive activity and an abundance of lymphoid tissue at this site.

TUBERCULOUS MENINGITIS

TBM accounts for 70 to 80 per cent of cases of neurological tuberculosis [41,54,51]. A majority of cases of TBM are caused by *M. tuberculosis*. Isolated cases of meningitis caused by NTM have also been documented86. Neurological tuberculosis is invariably secondary to tuberculosis elsewhere in the body. In the bacteraemic phase of primary lung infection, metastatic foci can get established in any organ, which can become active after a variable period of clinical latency. The critical event in the development of meningitis is the rupture of a subependymally located tubercle (Rich focus) resulting in the release of infectious material into the subarachnoid space[43]. The following features comprise the salient pathological features of TBM: (*i*) inflammatory meningeal exudate; (*ii*)ependymitis; (*iii*) vasculitis; (*iv*) encephalitis; and (*v*) disturbance of cerebrospinal fluid (CSF) circulation and absorption.

GENTIOURINARY TUBERCULOSIS

Genitourinary tuberculosis (GUTB) complicates three to four per cent of patients with pulmonary tuberculosis[19,52,61,30]. Haematogenous dissemination from an active site of infection results in GUTB. Initially metastatic lesions (tubercles) are formed in the kidneys. Macroscopic progression of the disease is often unilateral[24]. Usually, these lesions heal spontaneously or as a result of treatment. However, they may enlarge even

after years of inactivity and rupture into the nephrons producing bacilluria. There is descending spread of infection, inflammation and scarring. Active GUTB usually develops 5 to 25 yr after the primary pulmonary infection and is usually encountered between the second and fourth decades of life. Patients present with dysuria, haematuria which may be painless, flank pain, renal mass, sterile pyuria, and recurrent urinary tract infection. Rarely, acute presentation mimicking pyelonephritis has also

been described. Other uncommon presentations include: non healing wounds, sinuses or fistulae, haemospermia among others.

COLD ABSCESS

Scrofula is the term used for tuberculosis of the neck, or, more precisely, a *cervical tuberculous lymphadenopathy*. Scrofula is usually a result of an infection in the lymph nodes, known as lymphadenitis and is most often observed in immuno compromised patients (about 50% of cervical tuberculous lymphadenopathy). About 95% of the scrofula cases in adults are caused by *Mycobacterium tuberculosis*, but only 8% of cases in children. The rest are caused by atypical mycobacterium (*Mycobacterium scrofulaceum)* or nontuberculous mycobacterium (NTM). With the stark decrease of tuberculosis in the second half of the 20th century, scrofula became a very rare disease. With the appearance of AIDS, however, it has shown a

resurgence, and presently affects about 5% of severely immunocompromised patients.

SIGNS AND SYMPTOMS

The most usual signs and symptoms are the appearance of a chronic, painless mass in the neck, which is persistent and usually grows with time. The mass is referred to as a "cold abscess", because there is no accompanying local color or warmth and the overlying skin acquires a violaceous (bluish-purple) color. NTM infections do not show other notable constitutional symptoms, but scrofula caused by tuberculosis is usually accompanied by other symptoms of the disease, such as fever, chills, malaise and weight loss in about 43% of the patients.

Both during the initial (or primary) infection with TB and during any subsequent secondary active disease the bacteria are spread by blood or the lymphatic system to other parts of the body. In healthy people these bacteria are usually destroyed by the immune system. If some immune deficit is present some may concentrate at a particular site where they may lie dormant for years or even decades before causing disease.

The most common sites of infection for extra-pulmonary TB in order of frequency are

- Lymph glands

- Pleura (membrane than covers the lungs)
- Genito-urinary tract. In women uterine disease is probably the most common while in men the epididymis is the site most frequently affected. Both sexes are affected by renal or bladder disease equally.
- Bones and joints (also called osteotuberculosis)
- Meninges which may be rapidly fatal if not, treated in time
- Bowel and/or peritoneum
- Pericardium (membrane around the heart)
- Skin

DISSEMINATED TUBERCULOSIS

Disseminated TB, also referred to as miliary tuberculosis, is TB infection in several different organs simultaneously. Technically extra pulmonary TB is any TB infection outside the lungs, while miliary TB It differs from EPTB that is a widespread infection outside the lungs.

The symptoms of extra-pulmonary tuberculosis depend on the site(s) of infection. In lymphadenitis, the lymph glands become swollen painful and may have a rubbery texture.

Abscesses may form in the lymph glands and discharge onto the skin sinus giving a very unsightly combination of swelling and pus around the neck.

Osteo-TB or TB in the bones and joints, causes pain and swelling of the affected part. Spinal disease may cause paraplegia if enough of the vertebrae are destroyed to cause instability of the spine.

In tuberculous meningitis the symptoms usually begin gradually. Tuberculous meningitis may cause a wide variety of symptoms, including double vision and mental confusion developing over days or weeks. Patients may present with a headache that is either intermittent or persistent for 2-3 weeks. If not detected and treated coma may develop. If treated soon enough recovery may be complete but long term complications (sequelae) are likely if the treatment is delayed.

TB meningitis has the highest mortality of all complications of tuberculosis. Subtle mental status changes may progress to coma over a period of days to weeks. Fever may be low-grade or absent. Abdominal disease characteristically causes pain and constipation. If advanced it may cause complete obstruction of the bowel. Reported symptoms for genito-urinary TB include; flank pain, painful urination, or urination frequency problems. In men, genital TB may present as epididymitis or a scrotal mass. In women, genital TB may mimic pelvic inflammatory disease.

LABORTORY DIAGNOSIS

This can be broadly divided into

1) Demonstration by Direct microscopy - light and Fluorescent
2) Isolation of mycobacterium tuberculosis by culture
3) Serodiagnosis of the infection
4) Molecular methods

DIRECT MICROSCOPY -STAINING

i) ZIEHL-NEELSEN STAINING (ZN)

ZN staining is a highly specific technique where AFB is demonstrated after staining. It is positive only if number of AFB is more 10000 per ml of specimen. The correlation of positive smear to positive cultures may be only 25% to 40%.

ii) **SPECIAL STAINING FOR AFB**

FLUORESCENT MICROSCOPY

Auramine dye is a fluorochrome, which can be raised to a higher energy level after absorbing Ultra violet (Excitation) light. When the dye molecules return to their normal low energy state they release excess energy in the form of visible (fluorescent) light. Auramine requires blue excitation light, excitor filters that select light in 450-490 wavelength range and a barrier Fluorescent filter for 515. Brightly fluorescent bacilli appear Yellow against a dark background. Its sensitivity and specificity are fairly similar to ZN microscopy.

MYCOBACTERIAL CULTURE

Definitive diagnosis depends on the bacteriological isolation and identification of Mycobacterium species from clinical specimens.

SPECIMEN PREPARATION:

HOMOGENIZATION:

It is essential to release the mycobacteria from the body fluid or tissues in which they are embedded. Tissue requires mechanical homogenization before decontamination. N-acetyl -L Cysteine (NALC) or 4% Sodium Hydroxide (NaOH) is used as a mucolytic agent to assist liquefaction of purulent samples.

The high concentration of lipids in the cell wall of most mycobacteria makes them more resistant to killing by strong acid and alkaline solutions than other bacteria that may be present in the specimen. Consequently, specimens likely to contain a mixed bacterial flora are treated with decontaminating agent to reduce the undesirable bacterial overgrowth and to liquefy the mucus. After treatment with the decontaminating agent for a carefully controlled time period, the acid or alkali used is neutralized and the mixture is centrifuged at high speed to concentrate the mycobacteria

DIGESTION AND DECONTAMINATION:

Various agents such as 4% sodium hydroxide, trisodium phosphate alone or in combination with benzalkonium chloride (Zepheran), N-Acetyl L

cysteine (NALC), cetyl pyridium chloride (CPC),4% oxalic acid and 5% sulphuric acid can be used. The exposure time to these decontaminating agents is very critical, as overexposure leads to loss of considerable number of mycobacteria. Each mycobacteriologist should select the agents to be employed in his laboratory on the basis of the number and types of specimens received.

NEUTRALIZATION:

After the decontamination time of 15-20 minutes usually, the agents is neutralized by the addition of distilled water or phosphate buffer, in case of decontamination with NALC.

CENTRIFUGATION:

Carefully controlled centrifugal force is important in the recovery of mycobacterium from clinical specimens. This centrifugation is necessary to concentrate the bacilli. The sediment is inoculated into the media. The recovery of culture is increased when the relative centrifugal force is around 3000g for 15 minutes.

INOCULATION OF SPECIMENS:

Several egg based and agar based culture media are available for recovery of mycobacteria. Some of the non-selective media available are Lowenstein Jensen medium which is most commonly used in most of the clinical diagnostic laboratories, Petragnani medium, Middle brook 7H 10

agar and 7H 9 liquid medium. American Thoracic Society (ATS) medium, which contains less amount of malachite green, is recommended for use, for the usually sterile specimen such as CSF, pleural fluid, etc

For extra pulmonary tuberculosis, the specimen is inoculated in the selective Kirchner synthetic liquid medium containing horse or bovine serum is and Lowenstein Jensen Medium and is incubated at 37^{0} c . 10 -14 days is required for the growth in liquid medium and 3 to 6 weeks for the Growth in solid medium . It may yield high isolation from the paucibacillary samples. Subculture from the liquid medium on to the solid medium gives isolation of organism with distinct eugonic rough, tough and buff colonies. About 4 to 8 weeks is required for the growth.

AUTOMATED DETECTION SYSTEMS:

Many Semi-automated and automated mycobacteria detection systems are currently available; they are the radiometric Bactec system, non-radiometric MB/Bac T System and ESP Myco system. Other manual systems are the mycobacterium Growth Indicator Tube (MGIT) system and Septi-chek System.

IDENTIFICATION TESTS

These include Niacin production, Aryl sulphatase, Phosphatase, Catalase and Nitrate reductase activity. Classification of Mycobacterium into different species based on biochemical tests and growth characteristics have been described and evaluated by the International working group on Mycobacterial Taxonomy. The following basic tests are routinely employed to differentiate M. tuberculosis from other mycobacterial species.

IDENTIFICATION OF M. TUBERCULOSIS

Test	M. Tuberculosis	Others
Growth rate	Slow	Slow/rapid
Temp. Requirement	35^{o}C - 37^{o}C	25^{o}C – 37^{o}C
Pigmentation	Absent	Present/absent
Niacin	Positive	Mostly negative
Stability of catalase at 68^{o}C	Negative	Positive
Growth on PNB media	Negative	Positive

SPECIES LEVEL IDENTIFICATION OF NON-TUBERCULOUS MYCOBACTERIUM

RUNYON'S CLASSIFICATION

Runyon Group	Growth Rate	Pigmentation	Typical Member
I	Slow > = 5 day	Photochromogenic (Yellow or orange Pigment after exposure to light)	M. Kansasi M. marinum
II	Slow > 5 days	Scotscochromogenic (Yellow or orange pigment in the dark)	M. scrofulaceum
III	Slow > 5 days	Nonchromogenic No pigment in the dark or in the light	M. avium M. intracellulare
IV	Rapid < 4 days	Variable	Fortuitum complex M. smegmatis M. phelei

DRUG SUSCEPTIBILITY TESTS

The three general methods are in use.

THE ABSOLUTE CONCENTRATION METHOD:

It involves standard inoculum on media containing graded concentration of drugs and resistance is expressed in terms of MIC - minimum inhibitory concentration.

THE RESISTANCE RADIO METHOD:

Compares the growth of known strain in the same set and test strain is various dilutions of drug media and resistance is expressed in terms of ratio of MIC of test strain / MIC of standard strain.

PROPORTION METHOD

This method enables precise estimation of the proportion of bacilli, resistant to a given drug. If >1% of strain show resistance compared to control at a critical concentration, the strain is considered as resistant. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population tested.

LJ Slopes with different concentrations of various drugs are prepared and a standard inoculum giving approximately 10 CFU/ml is inoculated in to them and incubated at 37^{o}C. Cultures are examined for growth on 28^{th} day.

NONCONVENTIONAL AND NEW METHODS FOR DRUG SUSCEPTIBILITY TESTING

Drug susceptibility testing (DST) methods include the proportion method, the absolute concentration method, and the resistance ratio method. With the introduction of the BACTEC radiometric system, and its adaptation to perform DST of *M. tuberculosis*, these four methods were considered as the gold standard. The long turnaround time (TAT) and laboriousness of these methods stimulated the search for alternative and faster techniques. These new methods can be differentiated into genotypic and phenotypic methods.

GENOTYPICMETHODS

These methods search for the genetic determinants of resistance rather than the resistance phenotype. In general, they have two basic steps: a molecular nucleic acid amplification step, such as PCR to amplify sections of the *M. tuberculosis* genome, known to be altered in resistant strains and a second step of assessing the amplified products for specific mutations correlating with resistance. These methods have several advantages: a faster TAT (days instead of weeks), no need for growth of the organism, the possibility for direct application to clinical specimens, reduction of biohazard risks, and feasibility for automation. Unfortunately, they also have disadvantages, including problems with inhibitors when applying these methods directly to clinical samples.

DNA sequencing of PCR-amplified products has been the most widely used method, becoming the gold standard. It has been performed by both manual and automated procedures although the latter has been the most commonly used. It has been thoroughly used for characterizing mutations in the rpoB gene in rifampicin-resistant strains and to detect mutations responsible for resistance to other anti-TB drugs. Several other genotypic methods have been proposed to detect resistance to antibiotics, such as PCR-single strand conformation polymorphism, PCR-heteroduplex formation, and solid-phase hybridization assays. Moreover, the Line Probe Assay (LiPA-Rif)

based on the hybridization of amplified DNA from cultured strains or clinical samples to ten probes encompassing the core region of the rpoB gene of *M. tuberculosis*, immobilized on a nitrocellulose strip, and the Genotype mycobacterium tuberculosis test (MTB) DR (Hain, Germany), a commercial system for the detection of the *M. tuberculosis* complex and its resistance to rifampicin and isoniazid from culture samples based on the detection of the most common mutations in the rpoB and katG genes, respectively. The LiPA-Rif assay has been evaluated in different settings giving encouraging results.

DNA microarrays and real-time PCR techniques have also been proposed as alternative methods for drug resistance detection; the former still beyond the reach of clinical diagnostic laboratories, and the latter being increasingly evaluated with promising results.

PHENOTYPICMETHODS

New phenotypic methods assess inhibition of *M. tuberculosis* in the presence of antibiotics by detecting earlier signs of growth using various technologies, for example, the measurement of metabolism with the aid of color indicators, or oxygen consumption, by early visualization of micro-colonies, and by the use of phages.

The MGIT system, in its manual or automated version and based on the measurement of oxygen consumption, has been thoroughly evaluated for DST

of *M. tuberculosis* to first- and second-line drugs showing a good concordance with the gold standard proportion method.

The E-test, another commercial system (AB BIODISK, Solna, Sweden), based on strips with impregnated gradients of antibiotics for the determination of drug susceptibility allows the reading of minimal inhibitory concentrations directly on agar plates. Several studies have evaluated this test in comparison with the proportion method finding an agreement of >90 %. Two other commercial and automated methods for DST are the MB/BacT system (Organon Technika) and the ESP culture system II (Accumed International, Chicago, IL, USA). Both systems rely on heavy equipment and have also been evaluated in several studies.

SEROLOGICAL DIAGNOSIS

M. tuberculosis is the paradigm of the successful intracellular pathogen. Although the organism evokes both a humoral and a cellular immune response, it is the later that determines the outcome of an infection. A variety of immunodiagnostic tests for tuberculosis based on the recognition of specific host response to the infecting organism have been described. The first test was the tuberculin skin test. The short comings of this test include the inability to distinguish active disease from past sensitization and unknown predictive values . There has been development of serological tests

for tuberculosis, but no test has found widespread clinical use, because of low specificity.

Sensitivity and specificity increase if ELISA with purified antigen is done. The antigens tested in serological assays include the 38 KDa antigen, Lipoarabinomannan, Antigen-60, antigen 85 KDa complex and glycolipids including phenolic glycolipid Tb1, 2y3 – diacyl trehalose and lipo-oligosaccharide.

Most patients with tuberculosis produce antibody to glycolipids and 38 KDa and 85 complex antigens and most healthy controls do not. However, a small proportion of tuberculous patients still have low levels or an absence of antibodies against any of these antigens.

A number of antigen capture assays based on enzyme-linked I mmunosorbant assay, western blot analysis of M. tuberculosis $H_{37}RV$ culture filtrates antigen, radio immunoassay or agglutination of antibody coated latex particles have been described. There is wide variation in sensitivity and specificity of these tests and the antigen test cannot be recommended at this time.

MOLECULAR DIAGNOSIS

In recent times, polymerase chain reaction (PCR) has been found to be the most sensitive technique for rapid diagnosis of M. tuberculosis. This technique, capable of amplifying minute amounts of a specific DNA

sequence into millions of identical copies has revolutionalised molecular biology research.

PCR is accomplished in the following manner. A specimen that may contain the organism with the DNA sequence of interest is heated to denature the double stranded DNA. The specific synthetic oligonucleotide 'Primers' (short single stranded pieces of DNA) bind to the unique DNA sequences of interest and a heat stable DNA polymerase extends the primers to create a complete and complementary strand of DNA. This process is typically repeated sequentially 20-40 times, thereby creating million of copies of target DNA sequence. The amplified sequences can then be detected easily by gel elecrophoresis.

The IS 6110 belongs to a class of molecules known as transposons, which are self-replicating stretches of DNA. This sequence has been found in the M. tuberculosis complex of organism (M. Tuberculosis, M. africanum, M. microti and M. bovis) but in no other mycobacterial species. Thus IS6110 serves as a useful amplification target in diagnosing tuberculosis because except for M. tuberculosis the members of the M. tuberculosis complex are not the usual human pathogens or colonizers.

Other amplification targets for PCR are possible. A popular choice in molecular diagnostics has been to use the ribosomal RNA (r RNA) sequence as amplification target, as these sequences are found in abundance in living

organism and should allow even more sensitive PCR - based assay. Ribosomal RNA is highly specific and abundant for any particular species.

The Restricted Fragment Length Polymorphism (RFLP) technique using the IS6110 repetitive sequence is considered for typing the M.tuberculosis complex strains. It involves the extraction of genomic DNA, restriction endonuclease digestion with PvoII, Southern blotting and probing for IS6110. Standardization of the procedure facilitates inter-laboratory comparability of patterns. However comparison of profiles requires sophisticated software for image analysis and well-trained technical staff. Other molecular methods such as spoligotyping and whole genome finger-typing methods used for epidemiological studies. Other recent methods include phage-based assays (Commercial kits-FAST Plague-TB), which diagnosis TB directly from specimens. Newer Versions are also being developed for the detection of drug resistance directly from sputum specimens.

OTHER METHODS FOR RAPID IDENTIFICATION:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

Each species of mycobacterium appears to synthesize a unique set of mycolic acid. Qualitative and quantitative differences in the spectrum of mycolic acids present in the cell wall are reliable criteria to identify

mycobacterial species. Once mycobacterial cultures are available, the mycolic acids are extracted from saponified mycobacterium, converted to b - bromophenacyl esters and analyzed by higher performance liquid chromatography (HPLC). The resulting pattern is compared to a library of referring patterns to identify the species. This assay can provide rapid definite species identification for essentially any mycobacterium isolate in contrast to the time-consuming biochemical procedures and other conventional tests for speciation.

IMPLEMENTATION AND PERSPECTIVES FOR NEW DIAGNOSTIC METHODS

Concerning serological approaches for the diagnosis of TB, none of the several tests proposed until now using a variety of mycobacterial antigens have shown to be predictive enough to warrant their routine use as a diagnostic test for TB. Apparently, tests using a cocktail of antigens, rather than a single more specific antigen, have given better results. The new ELISA-based tests like the QuantiFERON-TB test and the T SPOT-TB assay, which measure the production of IFN-by activated T cells, are promising; however, more studies are needed in different settings to assess their usefulness as a diagnostic tool in certain populations, such as those subjects immune suppressed by HIV infection or other diseases, and in

children. The cost of these tests, since they are also available as a commercial kit, will have an impact on the feasibility for their implementation on a routine basis in the future.

The phage-based tests have also been evaluated in different settings either as a commercial kit or as the in-house method. The low sensitivity obtained in some of these studies could have been due to low infectivity of the phages, which also can be affected by the age and condition of the samples. In contrast, in the studies where the phage-based methods have shown an increased sensitivity as compared with direct microscopy and culture, the volume of sample used was up to five-times higher than that used for culture. It seems that, in their current format, the phage-based assays are not ready as a tool to improve diagnosis of TB. However, they seem to be appropriate for rapid rifampicin resistance detection.

MATERIALS AND METHODS

STUDY POPULATION

A total of 110 patients clinically diagnosed as Extra Pulmonary Tuberculosis attending as out-patient and as well as In-patient in Stanley Medical College and Hospital, Chennai were included in the study.

STUDY PERIOD

June 2009 to June 2010

PLACE OF STUDY

Stanley Medical Hospital and college Chennai

SPECIMENS

1. Cerebrospinal
2. Pleural Fluid
3. Ascitic Fluid
4. Pus
5. Urine

THE METHODOLOGY INCLUDED

- Collection of specimen.
- Specimen processing
- Identification of the pathogens
- Drug susceptibility tests

- Molecular Method for detection of Mycobacterium tuberculosis by polymerase Chain reaction from clinical specimens.

INCLUSION CRITERIA

All patients with suspected extra pulmonary tuberculosis were screened based on their clinical history and symptoms.

1. Age greater or equal to 18 years
2. Weight loss of at least 10% of healthy body weight.
3. One focal symptom lasting for two weeks or more [45,44,22,27,16,47].

EXTRA PULMONARY TB	
Tuberculosis meningitis (TBM)	Fever,Headache,Vomiting and alteration of sensorium
Pleural Tuberculosis	Fever ,dyspnea and Chest pain
Tuberculosis Peritonitis	Fever, ascitis and abdominal pain
Renal Tuberculosis	Flankpain, dysuria,hematuria and pyuria
Cold abscess	Fever, painless slowly progressive swelling, cervical lymphadenitis

EXCLUSION CRITERIA

1. Age below 18 years
2. Received Anti TB treatment within last 6 months.

SAMPLE COLLECTION

1. BODY FLUIDS

Cerebrospinal Fluid was collected by lumbar puncture. Pleural Fluid and Ascitic Fluid were collected in universal sterile container by aspiration aseptically. The larger specimen volumes were collected to increase culture yields.

Cerebrospinal Fluid	-	3 – 5 ml
Pleural Fluid	-	10 ml
Ascitic Fluid	-	10 ml

2. URINE SAMPLE

The first morning whole urine was collected on three consecutive days in sterile container.

3. PUS SAMPLE

Pus sample was aspirated from cold abscess in the cervical region using disposable syringe aseptically.

PREPARATION OF SMEARS: [58]

With the 5mm internal diameter24SWG nichrome wire, the specimen was taken and 3 X 2 cm wide smear was made in the center of the slide. Slides were air-dried and heat fixed in the safety cabinet (Class 1)[58].

FLUORESCENCE STAINING (AURAMINE PHENOL**):**[8]

- The slide was covered with freshly filtered auramine phenol for 10mts.
- Washed well with running tap water in a controlled flow.
- Decolourisation was done by covering completely with acid alcohol for 2mts.
- Washed again with running tap water.
- Counter stained with 0.1% potassium permanganate for 30 seconds.
- Washed again with water and was placed in a hot plate to dry.
- Examined the stained smear under the high power objective of the fluorescence microscope.

FLUORESCENCE MICROSCOPY:

The mercury vapour lamp was switched on 10 minutes before examining. Using the low power (magnification=x150) objective first examine a known positive slide to ensure that the microscope is correctly set up. Morphology of the bacilli was identified and was graded using the high power objective. With auramine staining, the bacilli appear as slunder with bright yellow fluorescent rods, standing out clearly against a dark background. A smear is positive it contains a minimum of 4 AFB of typical morphology in the entire smear. If less than 4 bacilli are present, report as negative, However, the number of bacilli, if less than four, is noted in the smear record book. For positive smears at least 50 fields have to be screened. Report the smear as negative if no AFB was seen in the entire smear.

No of acid-fast bacilli (AFB) per HPF	**Report**
No AFB- Entire Smear	Negative
< 6 bacilli per field	1+
6-100 bacilli per field	2+
> 100 bacilli per field or large clumps	3+

MODIFIED ZIEHL-NEELSEN STAINING PROCEDURE: [29]

1. The smear was dried and heat fixed.
2. The entire slide was flooded with strong carbol-fuchsin.
3 Intermittent heating was done until fumes arise for 5 minutes.
4. The slide was rinsed in a gentle stream of running water until all free stain is washed away.
5. The slide was flooded with acid alcohol (3%) as a decolorizing agent for 2 to 3 minutes.
6. Rinsed the slide thoroughly with water and drained the excess water from the slide.
7. Flooded the slides with (0.1%) methylene blue counter stain for 30 seconds.
8. Rinsed the slide thoroughly with water and allowed the smear to air dry.
9.Examined the stained smear under oil immersion microscope

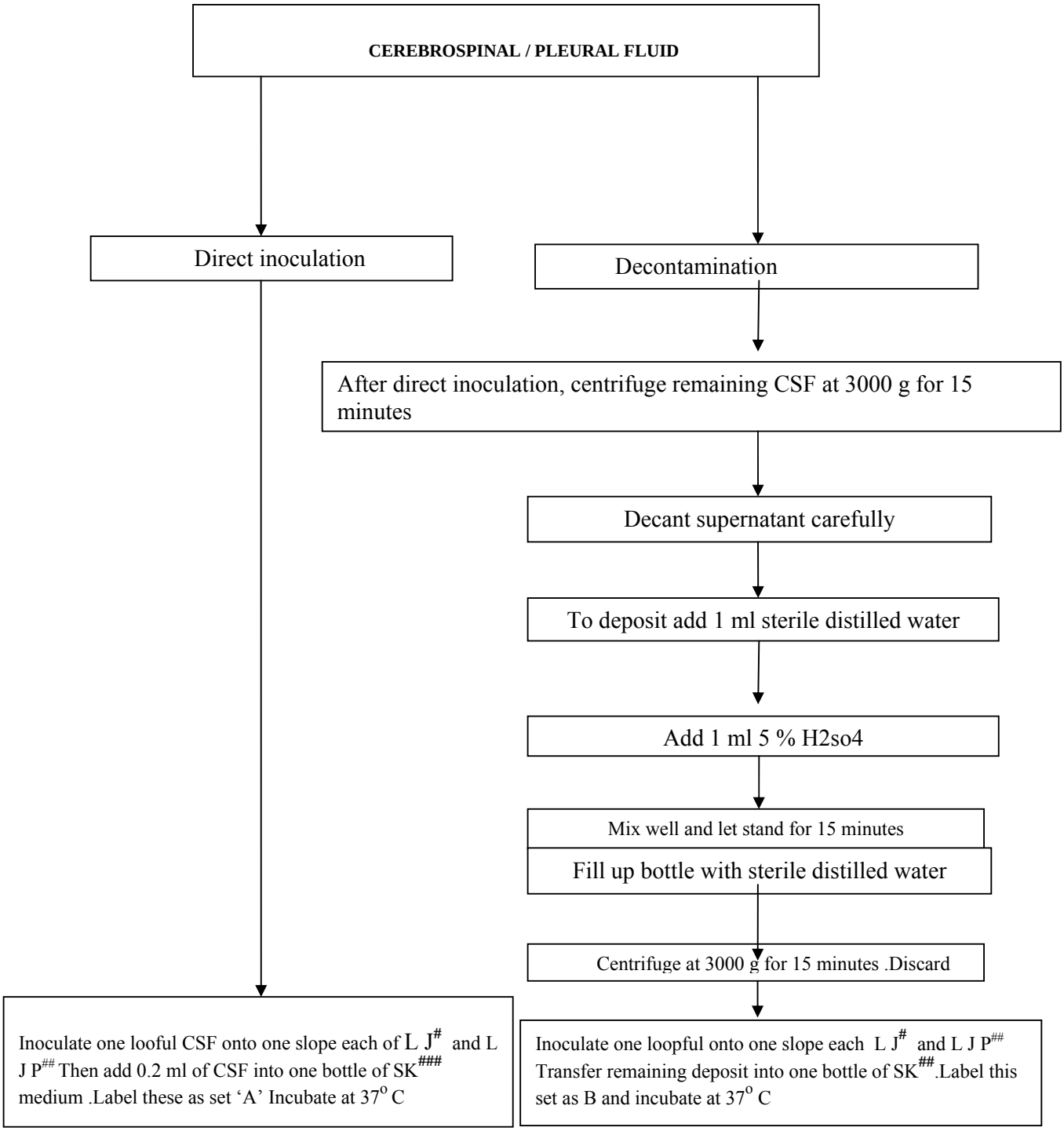
RECORDING AND REPORTING OF RESULTS: [20]

Ziehl-Neelsen's stained smears, were interpreted according to Tuberculosis research centre guidelines

No of acid-fast bacilli (AFB)	Report
No AFB/ 100 oil immersion fields	Negative
1-9 AFB/200 oil immersion fields	1-9 AFB per 200 fields
10-99 AFB/ 100 oil immersion fields	1+
1-10 AFB/ 50 oil immersion fields	2+
More than 10 AFB/ 20 oil immersion fields	3+

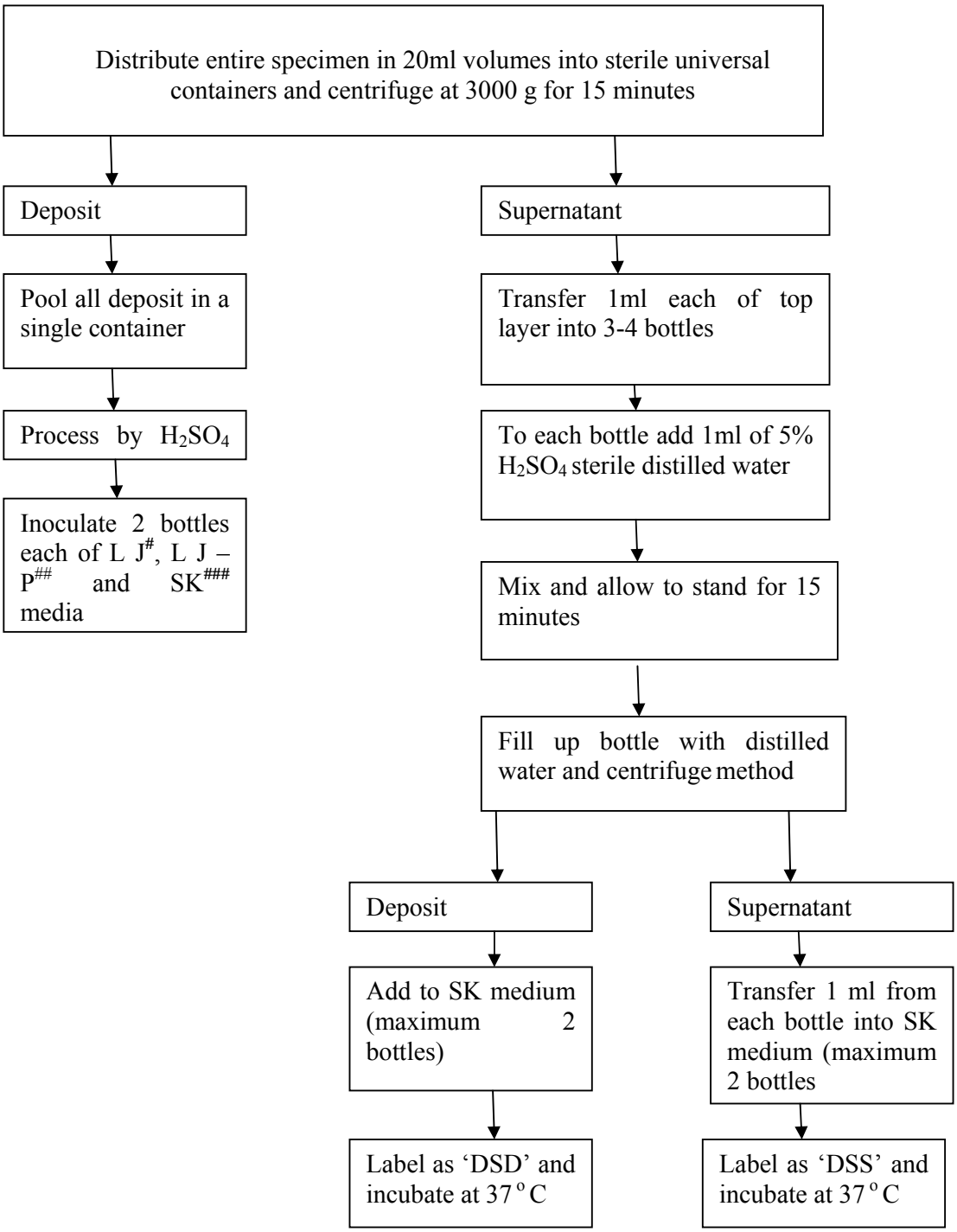
Culture Methods [3,33]

CEREBROSPINAL / PLEURAL FLUID PROCESSING



 # Lowenstein _ Jensen Medium
Lowenstien – Jensen Medium with sodium pyruvate
Kirchner"s Synthetic Medium

URINE /ASCITIC FLUID

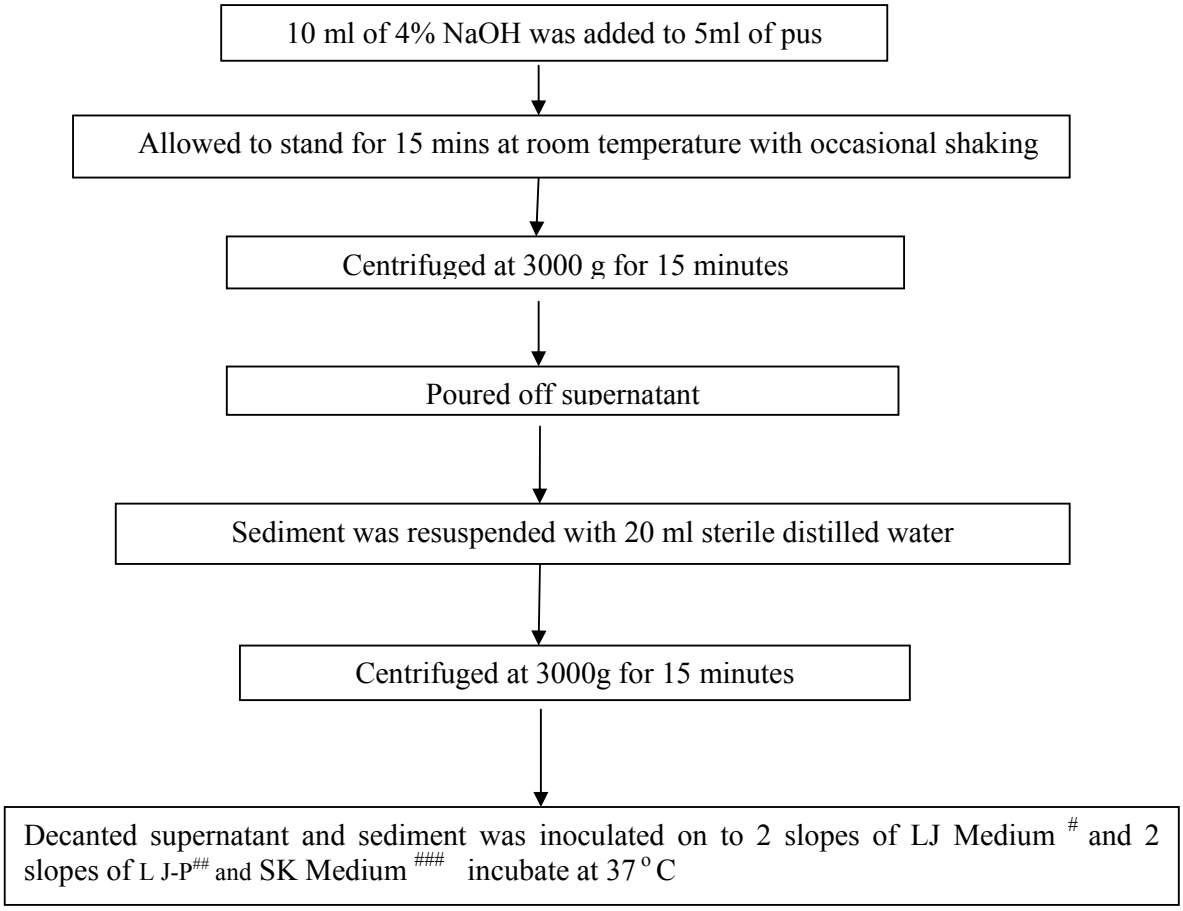


DSS - Decontaminated specimen Supernatant
DSD – Decontaminated specimen deposit

 # Lowenstien – Jensen Medium
Lowenstien – Jensen Medium with sodium pyruvate
Kirchner's Synthetic Medium

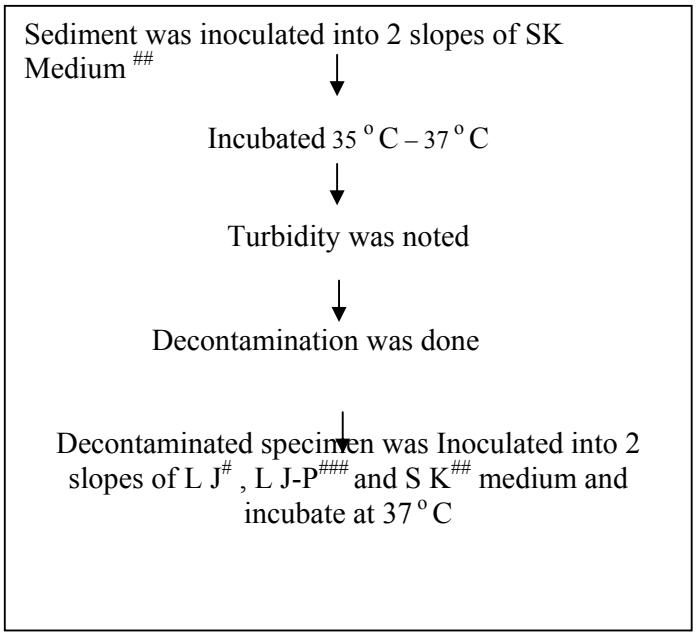
SAMPLE PROCESSING FOR PUS SAMPLES: [35]

The pus sample was homogenized in Vortex for few minutes to free bacilli from the mucus, cells or tissue in which they may be embedded. Decontamination was done by sodium hydroxide (modified Petroff) method.



 # Lowenstein _ Jensen Medium
Lowenstien – Jensen Medium with sodium pyruvate
Kirchner"s Synthetic Medium

PROCESSING OF SPECIMEN FROM LIQUID MEDIUM TO SOLID MEDIUM



 # Lowenstein _ Jensen Medium
Lowenstien – Jensen Medium with sodium pyruvate
Kirchner"s Synthetic Medium

PURPOSE OF CULTURE

Compared to other bacteria, which typically reproduce within minutes, M.tuberculosis proliferates extremely slowly (generation time 18-24 hours). Hence the cultures, which have been incubated at 37C for at least 9 days, are examined each Monday for 8 consecutive weeks or until they become positive or contaminated.

PRINCIPLE OF CULTURE

Tubercle bacilli do not grow in primary culture in less than one week and usually require two to four weeks to give visible growth from sputum specimens. Typical colonies of M.tuberculosis are rough, crumbly, waxy,

non-pigmented(buff colored) and slow-growers having the appearance of breadcrumbs or cauliflower.

CULTURE EXAMINATION AND IDENTIFICATION

All cultures examined 48 to 72 hours after inoculation to detect gross contamination. The cultures were examined weekly up to 8 weeks.

CULTURE REPORTS

Cultures were recorded qualitatively (growth positive or negative) as well as quantitatively (number of colonies isolated). The following scheme was recommended.

Reading	Report
No growth	Negative
1-19 colonies	Positive (number of colonies)
20-100 colonies	Positive (1+)
More than 100 discrete colonies	Positive (2+)
Confluent growth	Positive (3+)
Contaminated	Contaminated

IDENTIFICATION TESTS

Culture positives were identified based on the following test.

1. **Susceptibility to p-nitro benzoic acid (PNB)**
2. **Niacin test**
3. **Catalase activity at 68^{o}C/pH 7**

1. Susceptibility to p-nitro benzoic acid (PNB)

The neat bacterial suspension was inoculated into 2 slopes of LJ medium without drugs and 1 slope of LJ medium containing 500mg/liter of PNB and incubated at 37^{o}C. Reading was taken after 28 days.

Interpretation

M.tuberculosis does not grow on PNB medium.

2. Niacin test

Reagents

1. 1.5% Ortho -toludine
2. 10% Cyanogen bromide solution

LJ bottles with culture were Autoclaved at 121^{o}C for 20 minutes

↓

Bottles were placed upright for 20-30 minutes to cool and the fluid was allowed to drain to bottom

↓

Pipette out 0.25ml of the autoclaved culture extract into a clean screw-capped tube

↓

0.25ml of Ortho -toludine (1.5%) and 0.25ml of 10% cyanogen bromide solution was added sequentially to the culture tube.

↓

Pink color was observed within 5 minutes(+ve).

↓

Sufficient 4% NaOH solution was added to discard tubes for neutralization the cyanogen bromide.

Positive control: Extract from culture of M.tuberculosis H_{37} R_V.

Negative control: Extract from uninoculated tube of medium.

3.Catalse Test at 68^{o}C /pH7.0

REAGENTS

1. 0.067M phosphate buffer solution, pH7.0

   A. Na_2HPO_4 anhydrous 9.47g

   Distilled Water 1 litre

   Dissolved disodium phosphate in water to provide 0.067M solution (Solution1)

   B. KH_2PO_4 9.07g

   Distilled water 1 litre

   Dissolved in water to give 0.067 M KH_2PO_4 solutions (Solution B)

   Mixed 61.1ml of Solution A with 38.9ml of Solution2.
2. 30% hydrogen peroxide solution
3. 10% Tween-80

PROCEDURE

With a sterile pipette, aseptically added 0.5ml of 0.067 M buffer into 16 X 125 mm screw-capped test tubes

↓

Loopful of the culture was suspended in the buffer solution, using a sterile loop

↓

Tubes with the emulsified culture were placed in a previously heated water bath at 68^{o}C for 20 minutes.

↓

Tubes were removed from heat and cooled to room temperature

↓

Add 0.5ml of 10% tween 80 and 0.5ml of 30% hydrogen

↓

Formation of bubbles appearing on the surface of the liquid was observed. Tubes should not be shaken because Tween-80 may also form bubbles when shaken, resulting in false positives.

↓

Test tubes were kept for 20 minutes before discarding

Use a slope of M.tuberculosis as a negative control and M.terrae complex as a positive control.

INTERPRETATION

M.tuberculosis showed catalse negative at 68^{o}C.

SPECIES LEVEL IDENTIFICATION OF NON-TUBERCULOUS MYCOBACTERIUM :

- Mycobacterium fortuitum was identified by
    - growth on Mac Conkey Agar,
    - positive reaction for nitrate reduction,
    - growth on L-J medium with 5% sodium chloride
    - positive semi quantitative catalase test.
    - Iron uptake positive.
    - Sensitivity to ciprofloxacin.

- Mycobacterium chelonei was identified by
    - growth on Mac Conkey agar
    - negative reaction for nitrate reduction.
    - growth on L-J medium with 5% sodium chloride.
    - negative semi quantitative catalase test
    - Iron Uptake negative.
    - Sensitive to polymyxin

DETERMINING DRUG SUSCEPTIBILITY OF MYCOBACTERIUM TUBERCULOSIS BY PROPORTION METHOD [55]

PREPARATION OF MCFARLAND NEPHELOMETER BARIUM CHLORIDE STANDARDS (PAIK, G.1980)

a) Prepared 1% aqueous barium chloride (100mg of barium chloride (anhydrous) in 10 ml of sterile distilled water (SDW).
b) Prepared 10 ml of 1% sulphuric acid solution (99 ml of distilled water and 1 ml of concentrated sulphuric acid).

c) Added 0.1 ml of 1% Barium Chloride solution to 9.9 ml of 1% Sulphuric acid solution to obtain the McFarland standard, which matched with 1 mg/ ml of M. tuberculosis
d) Sealed the tubes (Wrap with Para film) and labeled as No. 1 McFarland standard tube with date of preparation
e) Used Wire meshes to hold McCartney bottles and bijou bottles

INOCULUM PREPARATION

1. With a 3 mm wire loop, a representative sample of approximately 4-5 mg (loop full) was taken from the primary culture and placed on the side wall of a McCartney bottle containing 1 ml SDW and 6 glass beads of diameter 3 mm
2. The bacterial inoculation was emulsified, (with a loop of water, if required), on to the side wall of McCartney bottle in round rotary movements with inoculation loop, till the bacterial mass was emulsified, (this was variable by reduction in the clumpy hydrophobic to aqueous hydrophilic nature of suspension)
3. The suspension was emulsified by fully dissolving in the 1 ml of sterile distilled water (SDW)
4. Vortex the bottle for 20-30 seconds
5. 4 ml of distilled water was added slowly
6. The coarse particles were allowed to settle down (leave it on stand for approximately 5 min)
7. The Mycobacterium solution was decanted carefully into another clear, sterile McCartney bottle
8. The turbidity of inoculums was matched with McFarland standard no.1, against a black background. This was the neat bacterial suspension standardized as 1 mg/ml, equaling to 10^7 to 10^8 CFU/ml.

9. If required, the opacity of the bacterial suspension was then adjusted by the addition of distilled water to obtain a concentration of 1 mg/ml of tubercle bacilli by matching with McFarland's standard
10. Make further two log dilutions were made to achieve 10^{-2} and 10^{-4} dilutions as given below

    a. The dilution 10^{-2} was produced by discharging two loop full of the neat bacterial suspension, into a small tube containing 2 ml of distilled water, and shaking
    b. Similarly the dilution 10^{-4} was produced by discharging two loop full of the dilution 10^{-2} into a small tube containing 2 ml of distilled water, and shaking

Neat: 1 ml SDW with six 3 mm glass beads + 1 loop-full (3mm Loop) of culture

↓

Vortex for 20 – 30 seconds

↓

Add 4 ml of SDW to the above

↓

Adjusted turbidity with McFarland std.1 with SDW

↓

S2-10^{-2} Two loop-full of **neat** + 2 ml of SDW, vortex
S2-10^{-4}

↓

Two loop –full of s2 + 2 ml of SDW

Preparation of Lowenstein-Jensen (LJ) Medium with anti-TB drugs (Streptomycin, Isoniazid, Rifampicin and Ethambutol)

All strains of tuberculosis contain some subpopulation of bacilli that are resistant to antiTB drugs. However, in resistant strains, the proportion of such bacilli is considerably higher than the sensitive strains. The proportion method calculates the proportion of resistant bacilli present in a strain. Two appropriate dilution of the bacilli, 10^{-2} and 10^{-4} dilutions (undiluted = 10^{6} to 10^{8} CFU/ml), were inoculated on drug-containing and drug-free media, in order to obtain countable colonies on both media. Numbers of colonies were observed on the drug-containing media to drug-free medium indicates proportion of resistant bacilli present in the strain. Below a certain proportion (critical proportion = 1%), the strain was classified as sensitive and above, as resistant.

$$\frac{\text{No of colonies appearing on drug containing medium}}{\text{No of colonies appearing on drug free medium}} * 100$$

< 1% Sensitive to the drugs.

>1% Resistance to the drugs.

One set of media bottles for testing one culture consist of five LJ slope, one for neat, two for 10^{-2} and two for 10^{-4} ; eight LJ drug containing slopes, two each for drugs H, R, E & S (One each for 10^{-2} and 10^{-4} suspensions) and one for p-nitro benzoic acid slope, were required.

H ISONIAZID

R RIFAMPICIN

E ETHAMBUTOL

S STREPTOMYCIN

Incubation and Reading

Incubated the inoculated slopes at 37^0C. Reading of the growth was taken at 28 days and again at 42 days.

Recorded the growth as Confluent growth = 3 +;

More than 100 colonies = 2 +;

Record actual number of colonies = 1 – 100 cols.

When the number of colonies on a given dilution was less than 5, counted the number of colonies with the next larger inoculums, or estimated if more than 100.

INTERPRETATION OF THE TEST

First reading was taken at 28^{th} day after inoculation.

1. Counted the colonies only on the slopes seeded with the inoculums that has produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculums may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculums (10^{-4} dilution) for the control slopes and the high inoculums (10^{-2} dilution) for the drug-containing slopes
2. The average number of colonies obtained for the drug-containing slopes indicated the number of resistant bacilli contained in the inoculums.

3. Dividing the number of colonies in drug containing slopes by that in drug free slopes gave the proportion of resistant bacilli existing in the strain. Below a certain value the critical proportion – the strain was classified as sensitive; above that value, it was classified as resistant. The proportions were reported as percentages.
4. If according to the criteria indicated below, the result of the reading was made on the 28th day was "resistant", no further reading of the test for that drug was required: the stain was classified as resistant. If the result at the 28th day was "sensitive" a second reading was made on the 42nd day only for the sensitive strain. The final definitive results for all four drugs on 28th day, then the report was given on the same day. Otherwise, incomplete reports should be given before 42nd day.

DEFINITION OF RESISTANCE

Drug	Level of Resistance
Streptomycin	Resistance ratio of drug conc. 8 μ g/ml or more were resistant
Isoniazid	MIC of drug conc. 5 μ g/ml or more were resistant
Rifampicin	MIC of drug conc. 128 μ g/ml or more can be tentatively interpreted as resistant
Ethambutol	MIC of 8 drug conc. 8 μ g/ml or more can be tentatively interpreted as resistant

MOLECULAR METHOD FOR DETECTION OF TUBERCULOSIS BY POLYMERASE CHAIN REACTION [10,14,12]

Extraction of DNA from clinical samples

DNAzol Direct is a universal reagent for processing biological samples for direct PCR. No isolation of DNA is required. The DNAzol Direct procedure is simple; lyse a sample in DNAzol Direct for 15 minutes, add an aliquot of the resulting lysate to a PCR mix and perform amplification of a selected DNA fragment(s). The standard DNAzol Direct procedure supports PCR amplification of DNA fragments up to 8 kb long.

The combined effects of the alkaline pH and chaoptropic properties of DNAzol Direct sufficiently inactivate PCR inhibitors including proteases and nucleic acid degradation enzymes. After processing a sample in DNAzol Direct, DNA is denatured into a single-stranded form, RNA is hydrolyzed, and proteins are denatured and partially hydrolyzed. Due to its unique composition, the DNAzol Direct lysate does not require neutralization before its use in PCR. The resulting pH of a PCR mix containing less than 10% of the lysate is within the effective range for PCR.

PROTOCOL

1. Mixed 10 µl of fluid sample with 0.1 ml(100 µl) of DNAzol Direct.
2. Lysed the sample by incubation in DNAzol Direct for 15-20 minutes at room temperature.
3. Vortexed the lysate and transferred a 3 µl aliquot directly into 22 µl of PCR mix.

PCR MIX[3]

1. 15 µl master mix
2. 1 µl forward primer
3. 1 µl reverse primer

4. 5 µl double distilled water
5. 3 µl DNA template

PCR AMPLIFICATION AND DOCUMENTATION

Amplification of DNA was performed with primers IS-F-5'-CCTGCGAGCGTAGGCGTCGG-3.and IS-R-5'CTCGTCCAGCGCCGCTTCGG-3., to amplify 123 bp fragment of insertion element IS6110 of *M. tuberculosis* complex as reported earlier,[2] . Amplifications was done. Conditions followed were initial denaturation at 94°C for four minutes, followed by 35 cycles at 90°C for one minute, 60°C for one minute, and 72°C for one minute, and a final extension at 72°C for 10 minutes. The amplified products were subjected to electrophoresis on a 2% agrose gel containing ethidium bromide and the results were documented. Throughout the PCR processing the three room procedure and other recommended stringent precautions were followed and the results were evaluated in the light of the performance of appropriate positive and negative controls, to avoid cross-contamination and false positive reactions.

Component	Vol./reaction	Final Conc.,
Taq Master Mix RED	25 µL	1X
Primer A	Variable	0.1-1.0 µM
Primer B	Variable	0.1-1.0 µM
Distilled Water	Variable	-------
Template DNA	Variable	Variable
TOTAL volume	50 µL	-------

PREPARATION OF AGAROSE GEL

- To prepare 2% agarose gel 400 grams agarose powder was mixed with 35 ml of electrophoresis buffer, heated in a microwave oven, mixed well until the agarose was uniformly dissolved.
- After cooling to about 60 °C, 10 micro litre of ethidiumbromide was added to the gel (final concentration 0.5 ug\ ml) to facilitate visualization of DNA after electrophoresis.
- After cooling the solution, it is poured into casting tray containing a sample comb and allowed to solidify at room temperature.
- After the gel hardness enough, the gel was mounted in electrophoresis tank.
- Electrophoresis buffer is poured into the electrophoresis tank so that the gel was completely immersed.
- The comb carefully removed .
- Ethidium Bromide is mutagenic and should be handled with extreme caution.

GEL ELECTROPHORESIS

- Electrical leads were connected. As the DNA amplified by PCR was charged negative, it migrates from cathode to anode.
- The mixture was slowly loaded into the slots of the submerged gel using a micropipette.
- Marker DNAs of known size was loaded into slots. Constant voltage of 50 -150 V was applied to allow the gel run until the

Taq DNA Polymerase Master Mix Red have migrated ¾ the length of the gel .

- All PCR products were analyzed in 2% agarose gel, stained with ethidium bromide and observed under UV transilluminator.

RESULT

The IS 6110 of Mycobacterium tuberculosis was amplified at 123 base pair.

RESULT

A total number of 125 clinically diagnosed EPTB patients based on symptoms and history were included in the study. The period of study was one year June 2009–2010. Specimens were collected from outpatients as well as inpatients of Government, Stanley Medical College and Hospital, Chennai. Among the 125 EPTB cases, 48 were Cerebrospinal fluid, 44 were pleural fluids, 15 were Ascitic fluids, 10 were Pus and 8 were Urine samples.

Samples were subject to standard bacteriological diagnosis by smear study (florescence staining & Ziehl –Neelson staining), culture examination by using solid Lowenstein-Jensen medium & Liquid Kirchner's medium and molecular method for detection of DNA of mycobacterium Tuberculosis by polymerase chain reaction using IS6110. Drug susceptibility testing done by proportion method .

TABLE 1

AGE DISTRIBUTION OF THE SPECIMEN CASES (N = 125)

SNo	**Age**	**Male**	**Female**	**Total**	**%**
1	18-30	29	16	45	36
2	31-40	23	17	40	32
3	41-50	17	5	22	17.6
4	52-60	10	1	11	8.8
5	61-70	7	0	7	5.6
TOTAL		86	39	125	100.00

The average age group in the study was 36.64.

TABLE 2

GENDER DISTRIBUTION OF THE CASES (N = 125)

S.No	**Sex**	**Numbers**	Percentage (%)
1	**Male**	**86**	68.8
2	**Female**	**39**	31.2
		125	100

The study group showed male predominance (68.8%).

TABLE 3

TYPE OF SPECIMENS (N = 125)

S. No.	Type of Specimens	No of Specimens	Percentage %
1	Cerebrospinal fluid	48	38.40
2	Pleural fluid	44	35.20
3	Ascitic fluid	15	12.00
4	Pus	10	8.00
5	Urine	8	6.40
	Total	125	100

Out of 125 samples, cerebrospinal fluids (48) and Pleural fluids (44) were predominant. The other fluids collected were Ascitic fluid (15), Pus (10) and Urine (8).

TABLE 4

SMEAR POSITIVES BY ZIEHL- NEELSEN STAINING (N = 125)

S.No	Types of samples	No of samples	Smear	
			ZN	%
1	**Cerebrospinal fluid**	**48**	**3**	6.25
2	**Pleural fluid**	**44**	**2**	4.55
3	**Ascitic fluid**	**15**	**1**	6.67
4	**Pus**	**10**	**2**	20.00
5	**Urine**	**8**	**2**	25.00
	Total	**125**	**10**	**8.00**

By Zeihl- Neelsen staining smear positives in Cerebrospinal fluid samples were three. In the Pleural fluid, Pus & Urine samples it was two and one in Ascitic fluid samples.

TABLE 5

SMEAR POSITIVES BY FLUORESCENCE STAINING (N = 125)

S.No	Types of samples	No of samples	Smear	
			FM	%
1	**Cerebrospinal fluid**	**48**	**4**	8.33
2	**Pleural fluid**	**44**	**3**	6.82
3	**Ascitic fluid**	**15**	**2**	13.33
4	**Pus**	**10**	**3**	30.00
5	**Urine**	**8**	**2**	25.00
	Total	**125**	**14**	**11.20**

Out of 48 Cerebrospinal fluid samples the smear positives by fluorescence staining were four. There were three positives in the Pleural fluids and Pus samples. Two smear positives were detected in Ascitic fluid and Urine samples.

TABLE 6

COMPARISON OF ZIEHL NEELSEN AND FLUORESCENCE STAINING

S. No	Types of samples	No of samples	Smear			
			ZN	%	FM	%
1	**Cerebrospinal fluid**	48	**3**	6.25	**4**	8.33
2	**Pleural fluid**	44	**2**	4.54	**3**	6.82
3	**Ascitic fluid**	15	**1**	6.66	**2**	13.33
4	**Pus**	10	**2**	20.00	**3**	30.00
5	**Urine**	8	**2**	25.00	**2**	25.00
	Total	**125**	**10**	**8.00**	**14**	**11.20**

Out of 125 samples, smear positives by Ziehl –Neelsen staining was found to be 10 and by fluorescence staining was 14. The percentage of smear positives by fluorescence staining was found to be greater than the Ziehl – Neelsen staining.

TABLE 7

Comparison of culture positive By KIRCHNER'S , LOWENSTEIN-JENSEN medium and LOWENSTEIN JENSEN with sodium pyruvate – p (n = 125)

S. No	Types of samples	No.of samples	LJ Medium	LJ With Sodium Pyruvate (LJ-P)	Time taken For Growth	Kirchners Medium	Time Taken For Growth
1	Cerebro Spinal Fluid	48	6	No Growth	3-4 wks	6	10-16 days
2	Pleural Fluid	44	4	No Growth -	3-4 wks	4	10-16 days
3	Ascitic Fluid	15	4	No Growth	3-5 wks	4	10-16 days
4	Pus	10	4	No Growth	2 days	4	2 days
5	Urine	8	4	No Growth	3 days	4	2 days
	Total	125	22			22	

The growth obtained in Kirchner's liquid medium was indicated by turbidity. The generation turn around period in liquid medium was 10 -16 days and by LJ medium in 21-42 days. Generation was faster in Liquid medium when compared to solid LJ medium.

TABLE 8

DISTRIBUTION OF MYCOBACTERIUM TUBERCULOSIS AND NON TUBERCULOUS MYCOBACTERIUM AMONG THE CULTURE POSITIVES

S. No	Types of Specimens	Total Specimens	Culture			
			TB	%	NTB	%
1	CSF	48	6	12.5	-	
2	PF	44	4	9.1	-	
3	AF	15	4	26.7	-	
4	PUS	10	-	-	4	40.0
5	URINE	8	1	12.5	3	37.5
TOTAL		125	15		7	

Mycobacteria tuberculosis isolated in cerebrospinal fluid were six, four in pleural fluid and Ascitic fluid, only one in the urine samples. There were four non-tuberculous Mycobacteria in pus sample and three in urine samples

TABLE 9

DISTRIBUTION OF SPECIES IN SPECIMEN POSITIVE FOR EXTRAPULMONARY TUBERCULOSIS

Specimen	SPECIES ISOLATED			
	M.tuberculos	M.fortuitum	M.chelonel	Total
Cerebro Spinal Fluid	6	-	-	6
Pleural Fluid	4	-	-	4
Ascitic Fluid	4	-	-	4
Pus	-	2	2	4
Urine	1	2	1	4
Total	**15**	**4**	**3**	**22**

Out 22 culture positives 15 samples were mycobacterium tuberculosis and seven were non tuberculous mycobacteria. Two of mycobacteria fortuitum each in pus and urine, two Mycobacterium chelonei were detected in pus and one in urine.

TABLE 10
RESULTS WITH
LJ CULTURE, Z-N STAIN AND FLUORESCENCE STAIN (N=125)

Result	**Z_N stain**	**Fluorescence stai**	**Culture positive**
Positive	10 (8.0%)	14 (11.20%)	22 (17.6%)
Negative	115 (92.0%)	111 (88.80%)	103 (82.4%)

The culture positives (17.6 %) found to be greater than

smear positives (11.2%).

Table 11

ANTIBIOGRAM

DRUG SUSCEPTIBILITY PATTERNS IN THE CULTURE POSITIVE CASES (n=22)

No. of Culture Positives	**Drug conc. for std. Sensitivity tests**				**PNB**	**Response to tests**
	Streptomycin	**INH**	**Rifampicin**	**Ethambutol**		
15	< 2μg	< 0.2μg	< 32	< 2μg	**Sensitive**	**Sensitive to all drugs**

PNB – Paranitro benzoic acid INH - Isoniazid

All the culture positive 15 samples found to be sensitive to first line drugs Streptomycin, Isoniazid, Rifampicin, Ethambutol

Table 12

PCR for Mycobacterium Tuberculosis

S.No	Types of samples	No of samples	PCR +ve	Percentage of PCR
1	Cerebrospinal fluid	48	14	29.16
2	Pleural fluid	44	10	22.72
3	Ascitic fluid	15	8	53.33
4	Pus	10	0	-
5	Urine	8	1	12.50
	Total	125	33	-

All culture positives were positive by Polymerase chain reaction. Eighteen culture negatives were detected as positive by PCR in addition.

Table 13

Results showing comparison of bacteriological investigation with Polymerase chain reaction using IS6110 primers coding for 123 bp of M.tuberculosis genome on various clinical specimens

S. No.	Type of Specimen	No of Specimen	Smear +ve/ Culture +ve	Culture +ve / Smear –ve	Smear –ve/ Culture –ve	PCR +ve
1	Cerebrospinal fluid	48	4	2	42	14
2	Pleural fluid	44	3	1	40	10
3	Ascitic fluid	15	2	2	11	8
4	Pus	10	3	1	6	0
5	Urine	8	2	2	4	1
	Total	125	14	8	103	33

Out of 125 specimen 14 smear and culture positives, 8 were culture positive and smear negative. Thirty three samples were found to positive by polymerase chain reaction 14 from Cerebrospinal fluid, 10 from Pleural fluid, 8 from Ascitic fluid and one from Urine. All culture positives are found to be positive by Polymerase chain reaction.

DISCUSSION

Despite the global importance of tuberculosis, the diagnosis of extra-pulmonary tuberculosis (EPTB) in its different clinical presentations remains a true challenge. Because of acid-staining and mycobacterial culture methods has low sensitivity for detecting EPTB, the development and evaluation of new diagnostic strategies needed for tuberculosis control programmes. The implementation of EPTB diagnostic methods with high specificity and sensitivity would improve the isolation of organism from EPTB patients, and should be accelerate the application of appropriate public health control measures.

The present study, a total number of 125 clinically diagnosed EPTB patients were included. The period of study was June 2009–May2010. Specimens were collected from outpatients as well as inpatients of Government Stanley Medical College and Hospital, Chennai. Among the 125 EPTB cases, 48 were Cerebrospinal fluid, 44 were pleural fluids, 15 were Ascitic fluids, 10 were Pus and 8 were Urine samples.

Samples were subjected to standard methods of diagnosis by smear study (fluorescence & Ziehl –Neelsen staining), culture on solid medium (Lowenstein-Jensen) & Liquid medium (Kirchner's) and molecular method by detection of DNA of mycobacterium Tuberculosis by polymerase chain

reaction using IS6110. Drug susceptibility testing by proportion method was done. The results obtained were discussed as follows.

The average age group in the study was 36.64, which correlated with the study done by Azar Dokht Khosravi et al[6] -37.8 years and Soumiesh Chakravarthy et al[48] and Shafi Ullah et al[48] - 35 years.

The present study showed male predominance (68.8%). The results of Meera Sharma et al[31] and L.Portillo-Gemez et al [37] **(55%)** also concorded with this study.

In this study cerebrospinal fluid 48(38.4%) and Pleural fluid 44 (35.2%) were predominant followed by Ascitic fluid 15 (12%), Pus 10(8.0%) and Urine 8 (6.0%). It correlates with the study of Lt Col KK Lahiri et al[25] showed out of 50 samples, cerebrospinal fluid 15(30%) and Pleural fluid 11 (22.0%) were predominant followed by Ascitic fluid 11 (22.0%), Pus 2(4.0%) and Urine 7 (14%)

By Zeihl- Neelsen staining smear positivity in Cerebrospinal fluid samples were three, in Pleural fluid, Pus & Urine samples it was two and one in Ascitic fluid samples. The smear positive by Ziehl-Neelsen staining was 8% . It correlates with Agartha Ani et al[2] – 6% and Rajeev Thakur[42] et al – 7.9%

Smear positive by fluorescent staining was 11.2 %. In the Indian study conducted by Gurung.R. Bhattachariya et al[38] showed 5.5%. The percentage of smear positive case by fluorescence staining was found to be greater when compared to Ziehl –Neelsen staining.

Growth was obtained in LJ medium by 21-42 days. Generation was faster in Liquid medium when compared to LJ medium. That correlated with study of Susmita Bhattacharya et al[50].

Out of 125 specimen fifteen cultures were positive for Mycobacterium tuberculosis among which 6(12.5%) were from cerebrospinal fluid correlate with Rajeev Thakur et al[42] showed 10.9 % culture positives and B Sekar et al[7] showed 17.3 %.

Out of 44 Pleural fluid samples the culture positivity for Mycobacterium tuberculosis was 9%, B Sekar et al[7] showed 12.5 %.

The Culture positives for Mycobacterium tuberculosis in Ascitic fluid was 27%. B Sekar et al[7] indicated 19.53 %.

Out of 8 Urine samples, culture positive for Mycobacterium tuberculosis was one (17.5%) which correlated Burkina Faso et al studied showed culture positive for Mycobacterium tuberculosis was 30.5% ,

There were four culture positives for non-tuberculousMycobacteria in pus sample 4(40%) which correlated with the study of M V Jesudasan et al[34] as 47%.

Out of 8 Urine samples, culture positive for non tuberculosis Mycobacteria was 3 (37.5%) which correlated Burkina Faso et al studied showed culture positive for non tuberculous Mycobacteria was 30.5% .

Among non tuberculous mycobacteria there were 4(18%) isolates of M.fortuitum and 3(13%) isolates of M.chelonei which correlated with study of M V Jesudasan et al[34] showed M.fortuitum was 35% and 42% of M.chelonei were isolated.

In the present study on comparing with staining, culture showed more positives (17.6%).

The following reference converges with our study:

B Sekar et al [7] - 18 %.

Agartha Ani et al[2] – 16%,

R.Gopal et al[40] --19.2 %.

All culture positives (15)were found to be sensitive to first line antituberculous drugs Streptomycin, Isoniazid, Rifampicin, Ethambutol.

All culture positives were found to be positive by IS6110 based PCR . 18 culture negative samples showed positive in PCR. PCR based assay showed more sensitivity than conventional methods. That correlated with study of MENG F. TAN et al[32] and Meera Sharma et al[6] .

As extra pulmonary specimens were paucibacillary in nature and culture takes a long time for growth, PCR assay targeting IS6110 was highly useful in the establishment of the diagnosis of EPTB.

To conclude PCR is a Rapid and Sensitive method in the detection of M.tuberculosis from extra-pulmonary specimens.

SUMMARY

- Total number of 125 cases of clinically suspected extra pulmonary tuberculosis were included in this study. Specimens were Cerebrospinal fluid, Pleural fluid, Ascitic fluid, Pus and Urine.
- Out of 125 samples 10 (8%) were found to be positive by Ziehl-Neelsen and fourteen (11.2%) by Fluorescence staining.
- Isolation of Mycobacterium tuberculosis was done by standard methods.The generation turn around period was 10-16 days in liquid medium (Kirchner's) and 21-42 days in solid medium (Lowenstein-Jensen).
- 22 samples were found to be positive by culture. 15 cultures were positive for Mycobacterium tuberculosis. 7 were positive for non tuberculosis Mycobacteria by standard phenotypic methods.
- Confirmation of Mycobacterium tuberculosis was done by susceptibility in L J medium with para-nitrobenzoic acid, Niacin test positive and positive reaction for nitrate reduction.
- M.fortuitum (4) and M.chelonei (3) were isolated among Non-Tuberculous Mycobacteria.

- ❖ All culture positive Mycobacterium tuberculosis were sensitive to first line antituberculous drugs -Streptomycin, Isoniazid, Rifampicin, Ethambutol.
- ❖ Mycobacterium tuberculosis genomes were found to be positive by Polymerase chain reaction using IS6110 primers coding for 123 bp from various clinical specimens.
- ❖ Culture positive for non tuberculosis mycobacterium were negative by polymerase chain reaction using IS6110 primers.
- ❖ Eighteen smear and culture negative were detected positive by polymerase chain reaction using IS6110 primers.

CONCLUSION

- A high index of suspicion is necessary to diagnose of Extra Pulmonary Tuberculosis
- Delay in diagnosis leads to sequelae in severe forms of Tuberculosis
- More than one diagnostic procedure is often needed for confirmation of the diagnosis, as Extra Pulmonary Specimens were paucibacillary in nature.
- Staining is a rapid method for detection of Mycobacterium Tuberculosis but always it should be confirmed with culture in Lowenstein-Jensen medium which is the Gold standard conventional method.
- Bacteriological and molecular methods aid in correct diagnosis of Extra Pulmonary Tuberculosis
- Drug susceptibility on culture positives will detect Multi Drug Resistant tuberculosis.
- Mycobacteria grow slowly and replicate every 18-20 hours, so growth in culture requires days or weeks. Culture is laborious and time consuming.
- The Rapid detection of bacilli with IS6110 based PCR assay is needed in management and prevention of complication.

MASTER CHART FOR EXTRA PULMONARY SPECIM

S.No	Patient	Age	Sex	Micro Id	OP/IP Id	Collection Dt	Specimen	Macroscopic	GRAMS	ZN	Fluorescence	BAP	MAC
1	Dass	42	male	454/09	18774/09	15-Jun-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
2	John F kennedy	40	male	472/09	19981/09	15-Jun-09	PF	CLEAR	No pus No Orgm-ve	**+ve**	**+ve**	No Growth	No Growth
3	Ebuneshwar	20	male	473/09	063006/09	15-Jun-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
4	Swetha	32	female	474/09	063106/09	15-Jun-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
5	Arul raj	25	male	475/09	19871/09	15-Jun-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
6	Muthuramalingam	55	male	677/09	6356/09	20-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
7	Mani	40	male	678/09	27319/09	20-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
8	Solaiyappa	62	male	666/09	27344/09	21-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
9	Kumar	31	male	682/09	2312/09	21-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
10	maaboobi	45	female	688/09	28166/09	24-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
11	Anwar	20	male	690/09	064488/09	27-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
12	Veerabathiran	18	male	694/09	28830/09	27-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
13	Arun	32	male	707/09	29050/09	28-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
14	Lakshmi	34	female	708/09	27261/09	28-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
15	Selvarani	38	female	702/09	28593/09	28-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
16	Chandra sekar	64	male	706/09	64137/09	28-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
17	Antony samy	42	male	709/09	276817/09	28-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
18	Vijay kumar	18	male	710/09	28759/09	28-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
19	Ponammal	38	female	712/09	29210/09	29-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
20	Mahendran	43	male	714/09	29411/09	29-Aug-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
21	Sangeetha	27	female	715/09	29381/09	29-Aug-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
22	bhavani	18	female	750/09	29855/09	7-Sep-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
23	viganesh	24	male	752/09	64783/09	7-Sep-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
24	saravanan	34	male	754/09	30134/09	8-Sep-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
25	Nithesh	45	male	755/09	64783/09	8-Sep-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
26	shanmugam	49	male	757/09	30271/09	8-Sep-09	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
27	Ramadas	55	male	758/09	24638/09	8-Sep-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
28	Balaganesh	29	male	800/09	31909/09	21-Sep-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
29	Amruthavali	31	female	801/09	31809/09	21-Sep-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
30	Murali	32	male	819/09	31867/09	21-Sep-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
31	Narayanasamy	33	male	820/09	32468/09	25-Sep-09	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
32	Marimuthu	56	male	875/09	32268/09	25-Sep-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
33	Dhakshi priya	21	female	876/09	34702/09	13-Oct-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
34	joshuwa	23	male	877/09	34722/09	13-Oct-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
35	Bharath	34	male	953/09	65485/09	10-Nov-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
36	hari krishanan	35	male	957/09	37909/09	10-Nov-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
37	Sudhakar	37	female	1031/09	42041/09	8-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
38	Raman	19	male	1032/09	42104/09	8-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
39	Kishore	22	male	1070/09	43837/09	12-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	**+ve**	No Growth	No Growth
40	yuvarani	45	female	1076/09	10930/09	16-Dec-09	URINE	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth

MASTER CHART FOR EXTRA PULMONARY SPECIM

S.No	Patient	Age	Sex	Micro Id	OP/IP Id	Collection Dt	Specimen	Macroscopic	GRAMS	ZN	Fluorescence	BAP	MAC
41	Selvaraj	41	male	1072/09	43539/09	23-Dec-09	CSF	CLEAR	No pus No Orgm-ve	**+ve**	**+ve**	No Growth	No Growth
42	Margret	35	female	1073/09	43801/09	23-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	**+ve**	No Growth	No Growth
43	Muniratinam	44	male	1075/09	43827/09	23-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
44	perumal	56	male	1079/09	43223/09	24-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
45	mangammal	60	female	1080/09	43845/09	24-Dec-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
46	Lakshmi narayanan	42	male	1083/09	43839/09	24-Dec-09	AF	CLEAR	No pus No Orgm-ve	**+ve**	**+ve**	No Growth	No Growth
47	ravi kumar	29	male	1099/09	44231/09	30-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
48	karthick	21	male	1100/09	43252/09	30-Dec-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
49	kesavan	47	male	1101/09	44267/09	30-Dec-09	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
50	doraikannan	40	male	1102/09	44270/09	30-Dec-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
51	ravi	47	male	1104/09	44281/09	30-Dec-09	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
52	kavitha	50	female	1106/09	44147/09	30-Dec-09	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
53	rengasamy	70	male	010/10	494/10	6-Jan-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
54	elavarasan	35	male	011/10	43878/10	6-Jan-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
55	kesavan	58	male	012/10	67169/10	6-Jan-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
56	venkatsamy	63	male	023/10	1056/10	11-Jan-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
57	sakrapani	40	male	067/10	3408/10	2-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
58	murgammal	40	female	070/10	3109/10	2-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
59	rajendran	55	male	072/10	3541/10	2-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
60	srinivasan	50	male	074/10	490/10	2-Feb-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
61	Lakshmi	19	female	088/10	4945/10	9-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
62	thirupati	64	male	090/10	4911/10	9-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
63	kalyani	32	female	138/10	6104/10	22-Feb-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
64	Vijaya	45	female	139/10	6609/09	22-Feb-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
65	moorthy	31	male	140/10	6621/10	22-Feb-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
66	vijaybaskar	39	male	141/10	6552/10	25-Feb-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
67	shanmugam	69	male	149/10	6707/10	25-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
68	manimaran	35	male	152/10	6756/10	25-Feb-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
69	Gokul	42	male	153/10	28977/10	25-Feb-10	CSF	CLEAR	No pus No Orgm-ve	**+ve**	**+ve**	No Growth	No Growth
70	Krishnaveni	38	female	158/10	6198/10	28-Feb-10	AF	CLEAR	No pus No Orgm-ve	-ve	**+ve**	No Growth	No Growth
71	vijaya lakshmi	28	female	101/10	2396/10	5-Mar-10	PUS	CLEAR	No pus No Orgm-ve	-ve	-ve	Opaque Colonies	Opaque Colonies
72	mallika	35	female	102/10	8643/10	8-Mar-10	PUS	CLEAR	No pus No Orgm-ve	-ve	-ve	Opaque Colonies	Opaque Colonies
73	manavalan	48	male	193/10	8653/10	9-Mar-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
74	mesanoori	21	female	201/10	8827/10	10-Mar-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
75	Simon	28	male	210/10	6502/10	12-Mar-10	CSF	CLEAR	No pus No Orgm-ve	-ve	**+ve**	No Growth	No Growth
76	raji	28	female	212/10	5126/10	13-Mar-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
77	narayanan	36	male	213/10	9054/10	15-Mar-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
78	palaniyappan	52	male	214/10	9073/10	15-Mar-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
79	sekar	46	male	236/10	10013/10	23-Mar-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
80	dinesh	24	male	238/10	10108/10	23-Mar-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth

MASTER CHART FOR EXTRA PULMONARY SPECIM

S.No	Patient	Age	Sex	Micro Id	OP/IP Id	Collection Dt	Specimen	Macroscopic	GRAMS	ZN	Fluorescence	BAP	MAC
81	sudarsan	24	male	239/10	10025/10	23-Mar-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
82	Chandra	40	female	252/10	30134/10	2-Apr-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
83	eshnakumar	48	male	282/10	12077/10	8-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
84	Nazeena beevi	32	female	288/10	61922/10	9-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
85	Radhakrishnan	48	male	294/10	11918/10	9-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
86	Rajadurai	19	male	297/10	12826/10	10-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
87	Priya	45	female	300/10	451365/10	10-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
88	Vjayalakshmi	30	female	311/10	11235/10	11-Apr-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
89	yusuf	30	male	313/10	13135/10	11-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
90	ragavah	67	male	318/10	12896/10	20-Apr-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
91	vanda	19	female	319/10	13523/10	20-Apr-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
92	paramaguru	24	male	320/10	08692/10	20-Apr-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
93	narayana	32	male	321/10	13633/10	20-Apr-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
94	mani kandan	32	male	324/10	22400/10	22-Apr-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
95	yuvaraj	22	male	325/10	13681/10	22-Apr-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
96	sabeetha	30	female	326/10	13679/10	22-Apr-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
97	Deepa	29	female	341/10	15348/10	11-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
98	Shanthi	29	female	342/10	15378/10	11-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
99	vasantha	32	female	350/10	14356/10	15-May-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
100	jyothi	23	female	361/10	14976/10	17-May-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
101	siddharth	27	male	362/10	62465/10	17-May-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
102	udayashakar	26	male	392/10	17020/10	17-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
103	veeraragavan	54	male	394/10	16900/10	17-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
104	thangavelu	55	male	395/10	16845/10	17-May-10	AF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
105	Govindasamy	57	male	403/10	17786/10	19-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
106	Parthiban	23	male	406/10	17858/10	19-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
107	Elumalai	45	male	404/10	17805/10	19-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
108	murugan	21	male	415/10	17458/10	27-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
109	Revathy	26	female	417/10	62978/10	27-May-10	CSF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
110	Gopal	20	male	418/10	18348/10	27-May-10	PF	CLEAR	No pus No Orgm-ve	-ve	-ve	No Growth	No Growth
111	Kala	31	female	11088/09	6519/09	18-Dec-09	URINE	CLEAR	No pus No Orgm-ve	+ve	+ve	No Growth	Growth
112	Purushothaman	30	male	1135/09	2804/10	26-Dec-09	URINE	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
113	Venkatesan	30	male	60/10	2213/10	4-Jan-10	URINE	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
114	Jayaraman	36	male	151/10	2415/10	6-Jan-10	URINE	CLEAR	No pus No Orgm-ve	+ve	-ve	No Growth	Growth
115	Santhi	35	female	336/10	961/10	8-Jan-10	URINE	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
116	Govindasamy	40	male	1105/10	4126/10	4-Feb-10	URINE	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
117	Sundar	40	male	137/10	15761/10	7-May-10	URINE	CLEAR	No pus No Orgm-ve	+ve	-ve	No Growth	Growth
118	Ravi	40	male	957/10	1696/10	1-Apr-10	PUS	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
119	Suresh	29	male	955/10	1082/10	1-Apr-10	PUS	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
120	Shanmugam	22	male	991/10	3023/10	6-Apr-10	PUS	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth

MASTER CHART FOR EXTRA PULMONARY SPECIM

S.No	Patient	Age	Sex	Micro Id	OP/IP Id	Collection Dt	Specimen	Macroscopic	GRAMS	ZN	Fluorescence	BAP	MAC
121	Neelan	40	male	1008/10	9288/10	8-Apr-10	PUS	CLEAR	No pus No Orgm-ve	+ve	-ve	No Growth	Growth
122	Paramesvari	21	female	1183/10	13983/10	23-Apr-10	PUS	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
123	Madhan kumar	20	male	1433/10	16541/10	13-May-10	PUS	CLEAR	No pus No Orgm-ve	+ve	-ve	No Growth	Growth
124	Valliammal	36	female	1459/10	13687/10	15-May-10	PUS	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth
125	Mala	30	female	1513/10	17105/10	21-May-10	PUS	CLEAR	No pus No Orgm-ve		-ve	No Growth	No Growth

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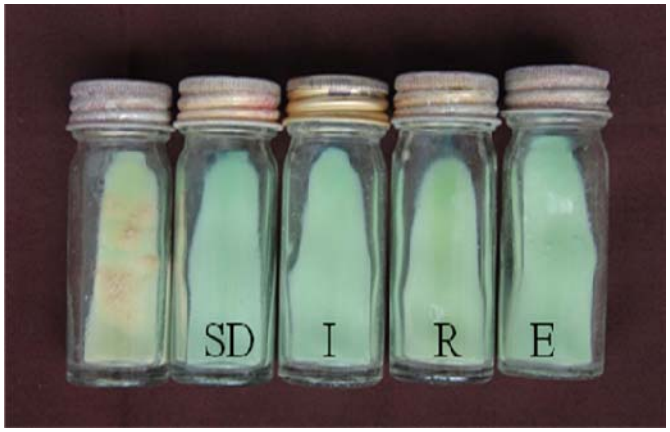
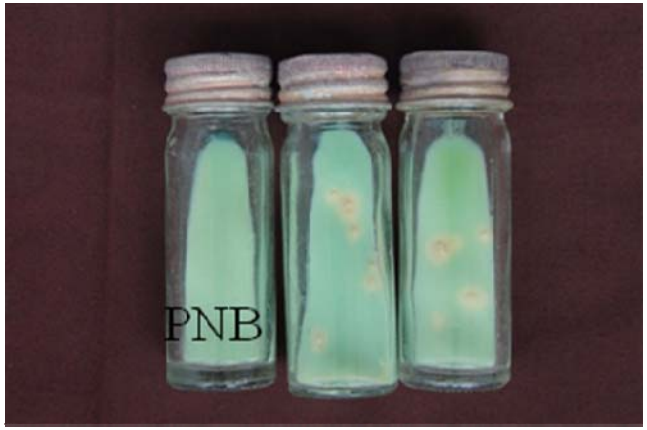
DRUG SENSITIVITY BY PROPORTION METHOD

S2 dilution



S3 dilution

S4 dilution



SD – Dihydrostreptomycin 4mg/l

I – Isoniazid 0.2mg/l

R – Rifampicin 40mg/l

E – Ethambutol 2mg/l

PNB – LJ with Para Nitro Benzoic Acid

Isolate of Mycobacterium tuberculosis was sensitive to First line drugs.

Table 2
GENDER DISTRIBUTION

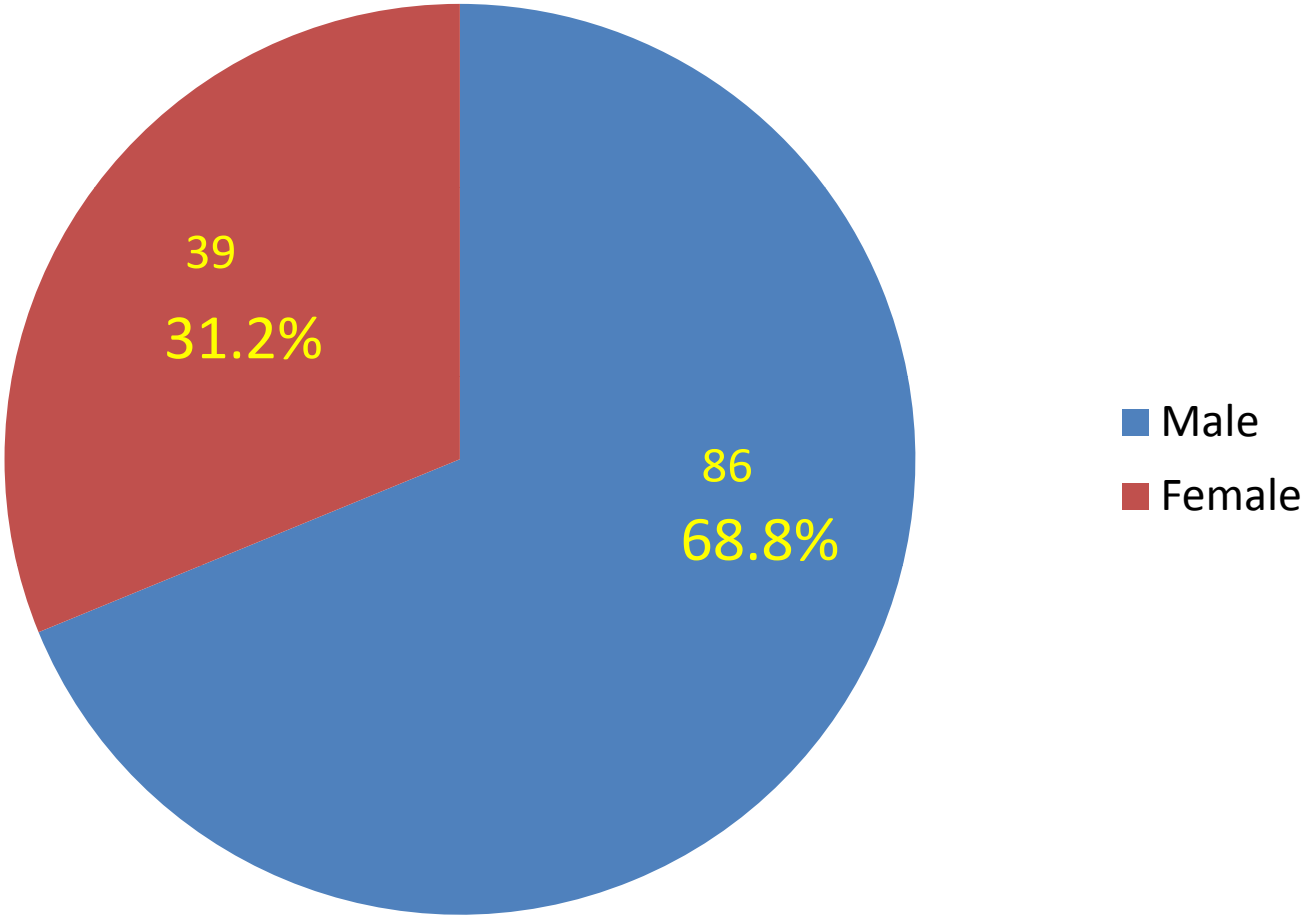


Table 3
TYPE OF SPECIMENS

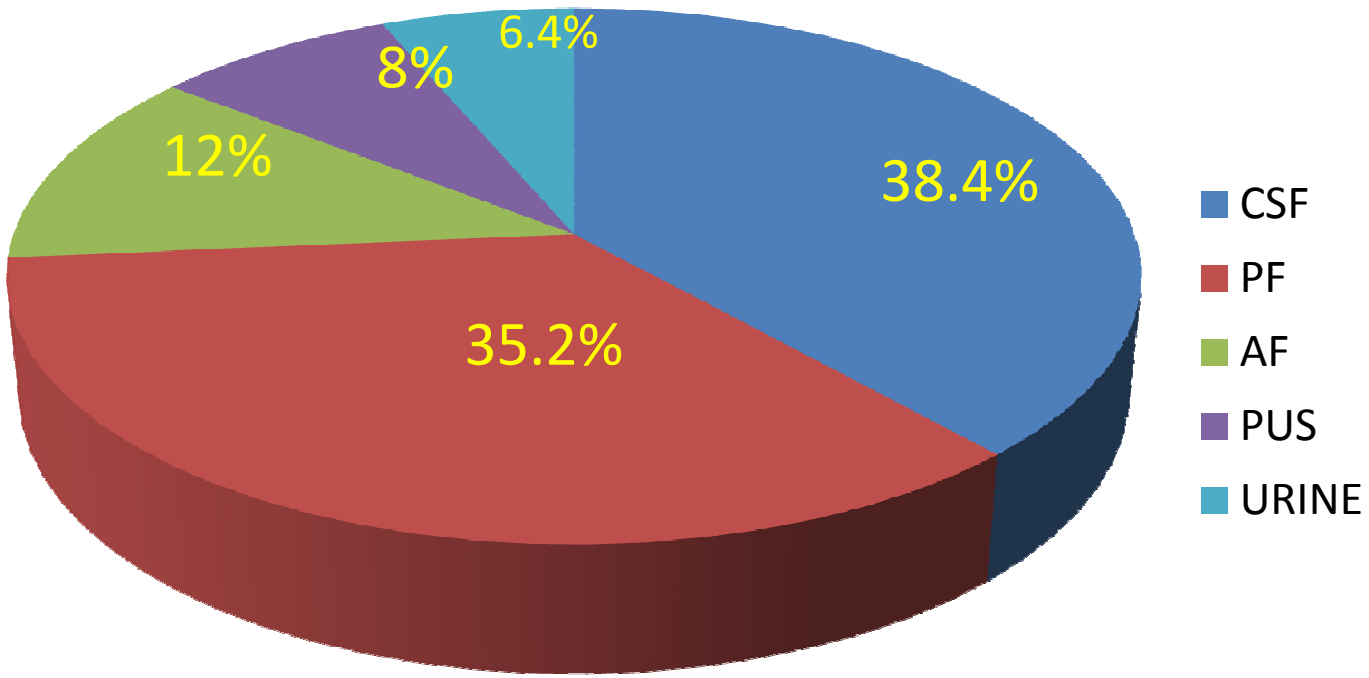


Table 4
Smear Positive by Ziehl Neelsen Staining

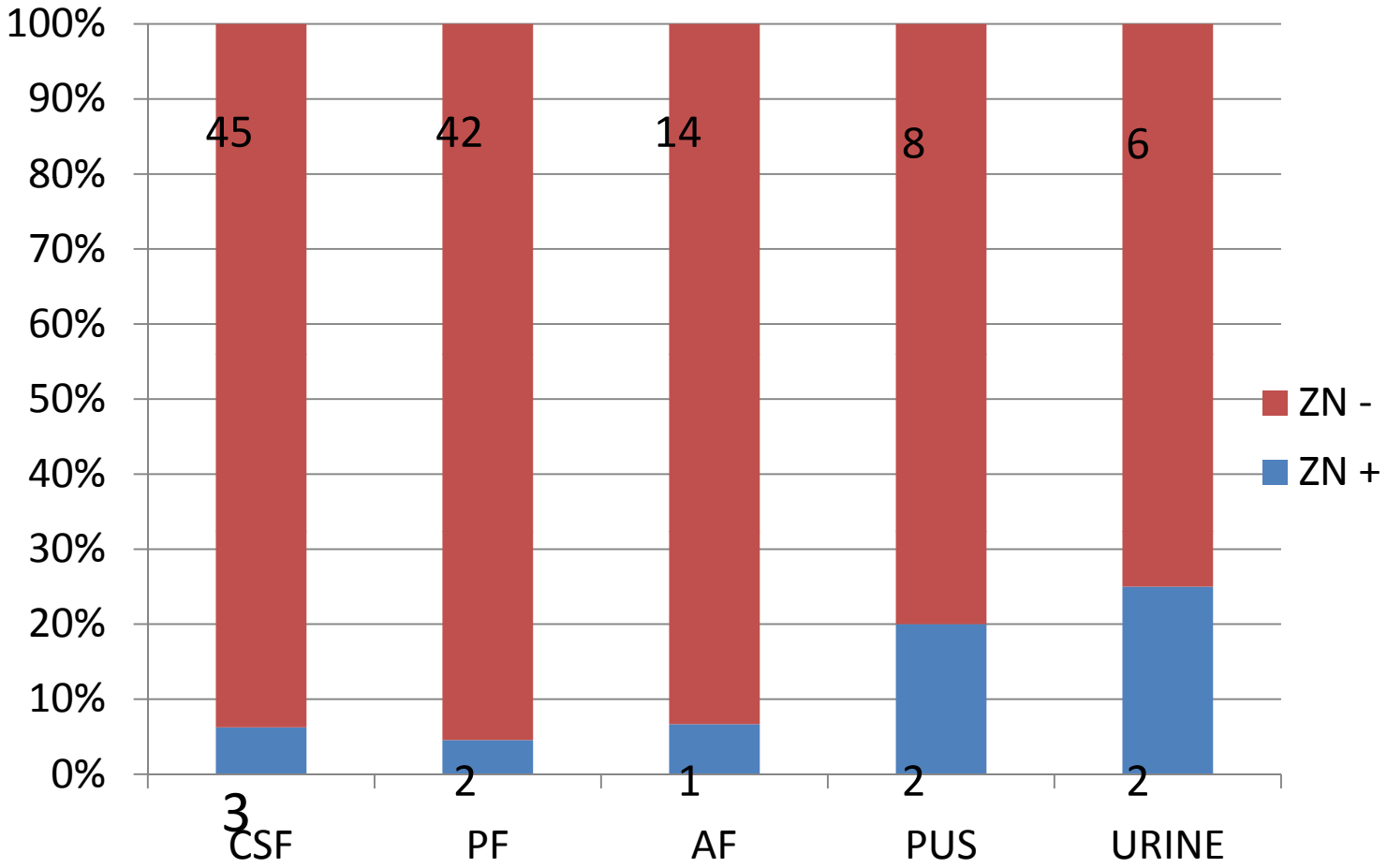


Table 5
Smear Positive by Flourescence Staining

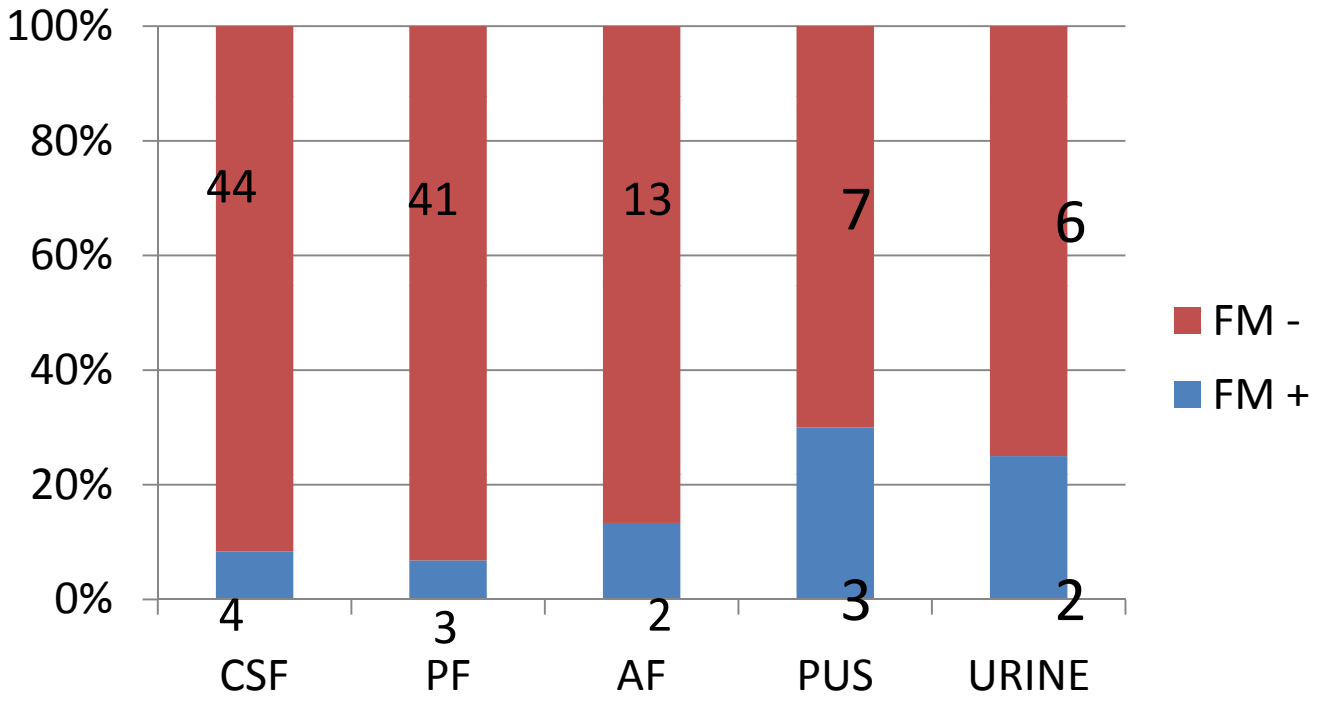


Table 7

Comparison of Culture Positive by Kirchner's and Lowenstein-Jensen Medium

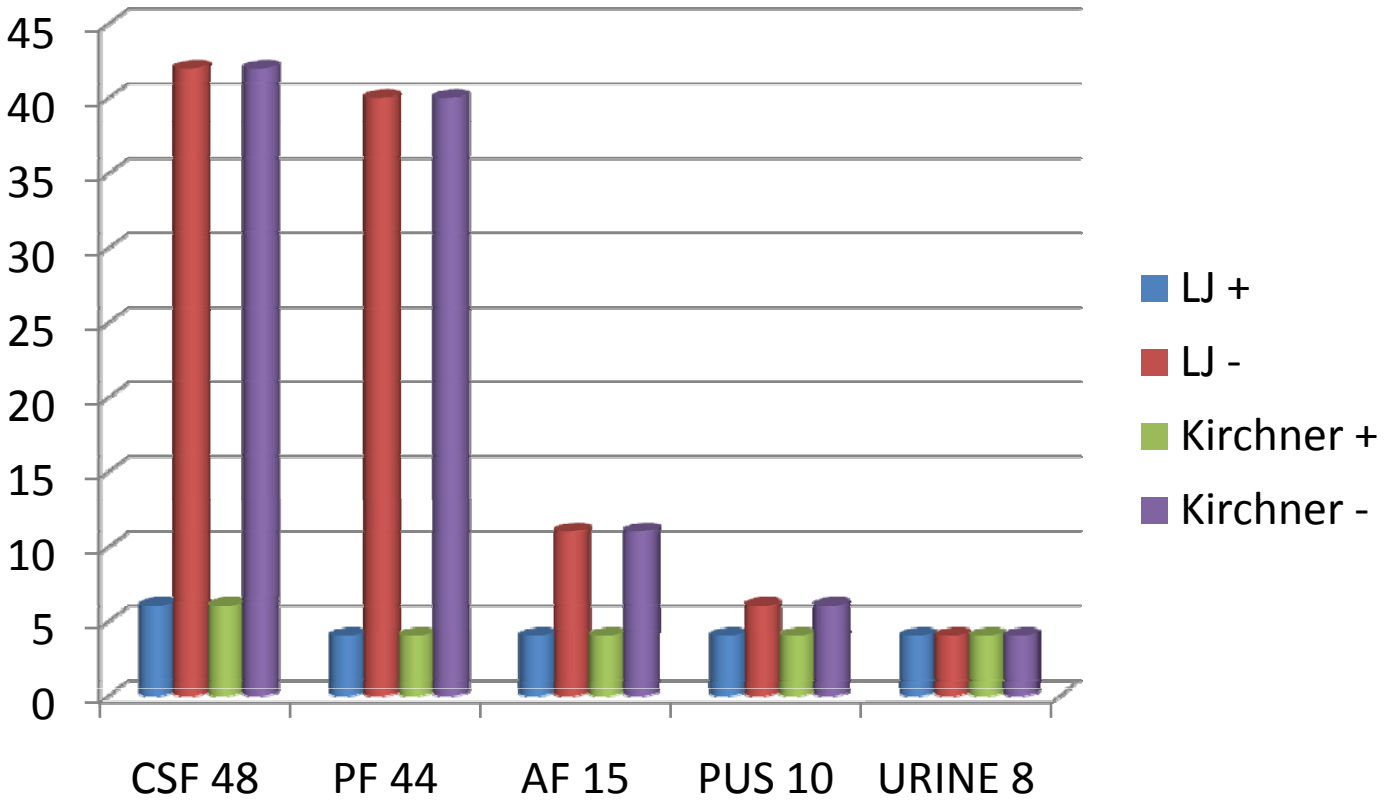


Table 10

Results with LJ Culture, Z-N stain and Fluorescence Stain(n=125)

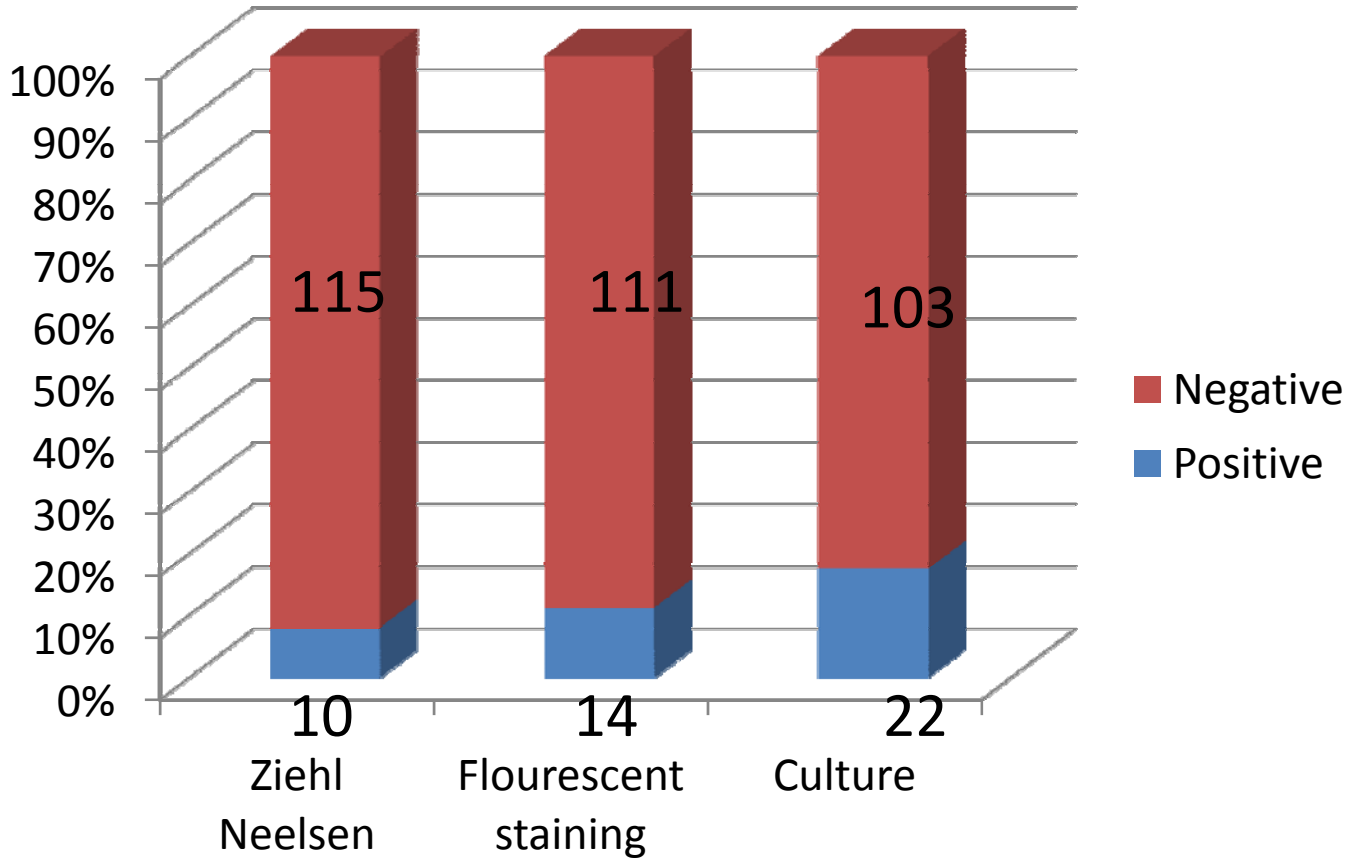


Table 8

Distribution of Mycobacterium Tuberculosis and Non Tuberculosis Mycobacteria Among the culture positives

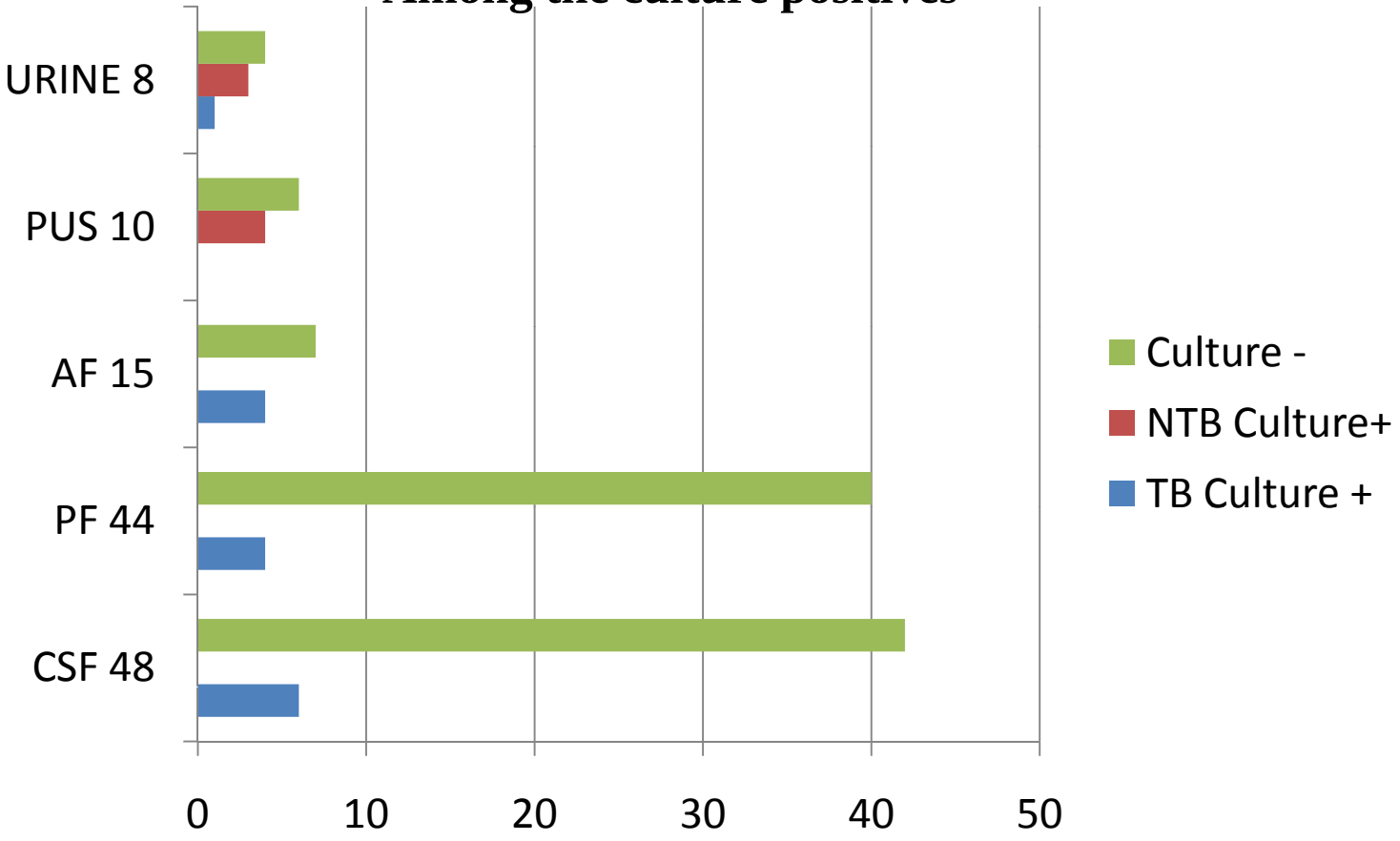


Table 9

Distribution of Species in specimen positive for extra-pulmonary tuberculosis

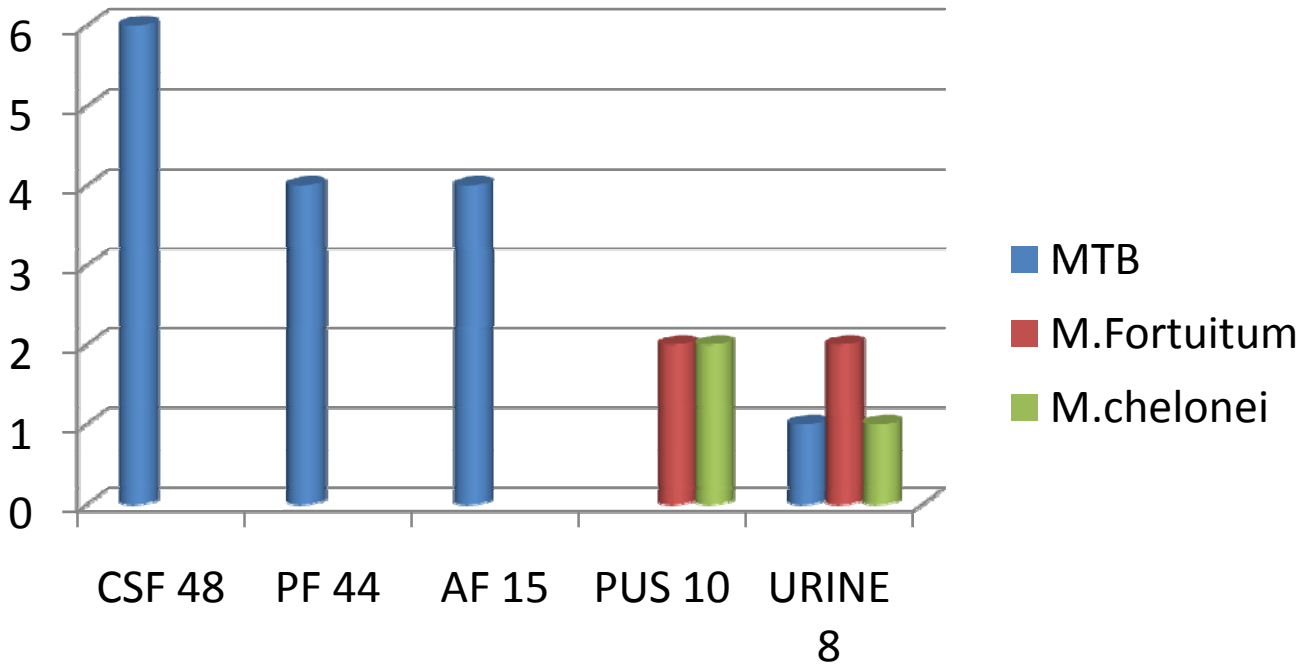


Table 13

Results showing comparison of bacteriological investigation with Polymerase chain reaction using IS6110 primers coding for 123 bp of M.tuberculosis genome on various clinical specimens

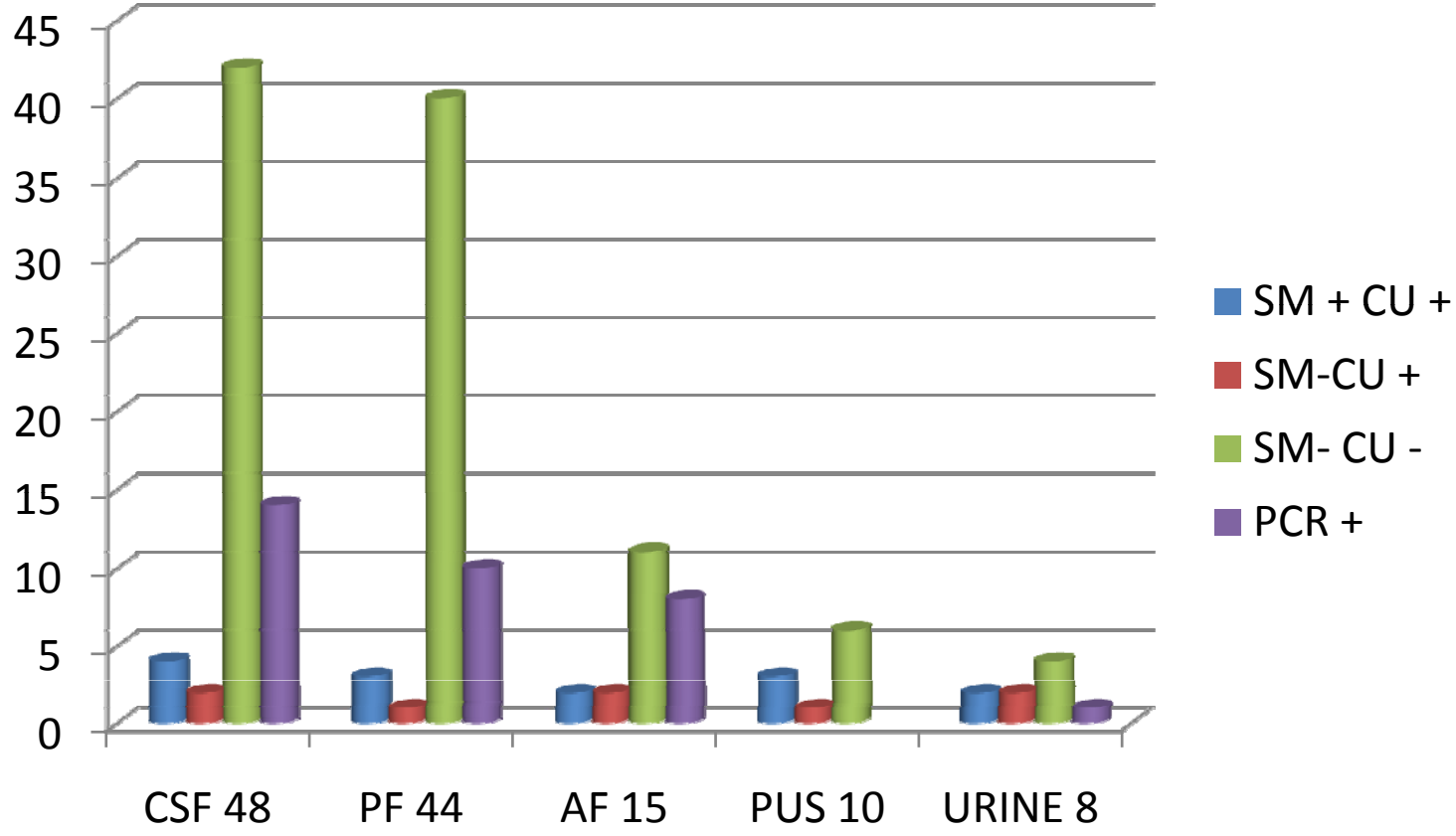
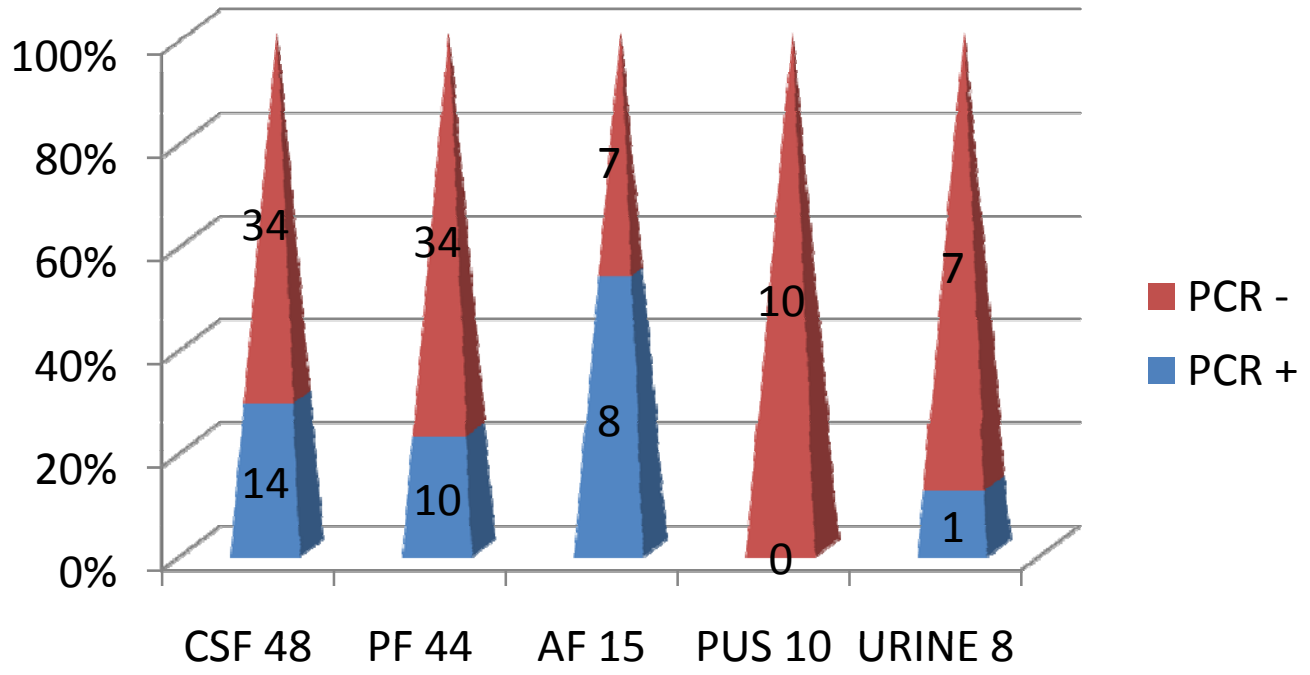


Table 12

PCR for Mycobacteria Tuberculosis



DRUG SENSITIVITY BY PROPORTION METHOD

Agarose Gel Electrophoesis Of IS 6110 Based Polymerase Chain Reaction For Detection Of M.Tuberculosis From Clinical Specimens